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(57) Abstract

A method for isolating a cDNA specific for the human ryanodine receptor is disclosed. The gene is associated with malignant hyperthermia, a hypermetabolic syndrome triggered primarily by inhalation anaesthetics, but also present in animals, such as pigs, as induced by stress. The cDNA can be cloned and expressed in a recombinant plasmid or phage. The cDNA, or fragments thereof, is used as diagnostic probes for individuals or animals at risk for malignant hyperthermia using restriction fragment length polymorphism analysis.

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DIAGNOSIS FOR MALIGNANT HYPERTHERMIA

FIELD OF THE INVENTION

This invention relates to the animal disease malignant hyperthermia (MH) and to the cloning and characterization of a gene associated with (MH), and to the development of methods for detecting individuals susceptible to MH.

BACKGROUND OF THE INVENTION

Although malignant hyperthermia is associated primarily with humans and was first realized as a reaction to inhalation anaesthetics, it is understaood that MH is also a very common problem in certain animals, particularly pigs. There is therefore particular commercial interest in developing suitable assays to determine MH in pigs, as well as providing suitable diagnosis to test humans to avoid life threatening circumstances in the operating room.

Malignant hyperthermia (MH) is an inherited predisposition to a hypermetabolic syndrome (adverse reaction) triggered by inhalation anaesthetics such as halothane and some skeletal muscle relaxants such as succinylcholine. The primary defect in MH is related to a sustained increase in myoplasmic calcium which causes muscle contracture and increased glycolysis concomitant with the production of H_2O, CO_2 and heat and excessive consumption of O_2. Other signs of the disorder (including the hyperthermia for which it was named) may be explained as a direct result of muscle contracture and increased glycolysis [Steward, D. J. and O'Connor, G. A. R, "Malignant Hyperthermia - The Acute Crisis", in Britt B. A. ed. Malignant Hyperthermia, Boston, Martinus Nijhoff, (1987)].

The disease in humans is a serious health problem as the affected individuals are usually unaware of their condition and problem with a potentially lethal reaction to the drugs administered at surgery. The observed frequency of the disease is d pendent on the drugs

administered. The highest estimate based on the use of succinylcholine in combination with halothane, gives a frequency f 1 in 4200 anaesthetics [Ording,H., Anesth Analg. 64: 700-704, (1985)]. This may be a gross underestimate of the true gene frequency since many individuals who carry the gene are never exposed to the triggering agent and thus remain undiagnosed. Additionally the incidence of masseter muscle spasm following halothane-succinylcholine induction of anaesthesia is 1 in 200; 50% of these individuals have been subsequently shown to have biopsies positive for MH suggesting that the true incidence of the trait may be considerably higher [Rosenberg, H, and Fletcher, J. E., Anesth. Analg. 65: 161-164, (1986)]. In many families the disease segregates as an autosomal dominant condition [Kalow, W., "Inheritance of Malignant Hyperthermia - A review of Published Data", in Britt, B. A. Ed. Malignant Hyperthermia, supra, (1987)] although other modes of inheritance have been reported in some families.

The mortality rate for MH in North America has decreased from 84% in the 1960s to about 7% [Britt, B. A., "Preface: A History of Malignant Hyperthermia", in Britt B.A. ed. Malignant Hyperthermia, Boston, Martinus Nijhoff, pp 1 - 10, (1987)], following improvements in monitoring systems, increased awareness of MH and the advent of dantrolene treatment in 1975. A marked elevation in end-tidal (exhaled) carbon dioxide levels is an early indicator of an MH reaction and,where monitored and recognized, may allow prompt treatment with sodium dantrolene (dantrium) to avert a full crisis. The fatality rate is still unacceptably high in many countries in the world (e.g. 24% in the U.K.).

Fatalities may result from one or more of multiple complications in a fulminant MH crisis. Skeletal muscle, smooth (involuntary) muscle and cardiac muscle are all affected in an MH reaction. Contraction of smooth muscle of the blood vessels causes hypertension which further

decreas s the oxygen supply and results in accelerated deep breathing. Pulmonary edema may occur as the crisis progresses especially at the onset of cardiac failure. Cardiac failure is triggered both by rigidity of the heart muscle and by elevated levels of potassium in the blood. Once the temperature of an affected individual has begun to rise it does so rapidly (1°C every five minutes) and final temperatures as high as 46°C have been reported. Leakage of myoglobin into the blood as a result of membrane damage may trigger kidney failure in survivors. Some survivors never regain consciousness and others have central nervous system damage (e.g. paralysis, blindness, deafness, impaired intelligence, speech defects) as a result of extremely high fever and/or electrolyte imbalance [Steward D.J. and O'Connor, G. A. R. supra, (1987)].

Accurate laboratory tests are required which can detect individuals at risk for developing malignant hyperthermia. Currently, the best test for individuals at risk is a diagnostic muscle biopsy. This test, described by Kalow et al. ["Metabolic error of muscle metabolism after recovery from malignant hyperthermia", Lancet, 2: 895-898, (1970)], is based on the abnormal contracture response of MH muscle to caffeine, halothane and a combination of the two. It is a highly invasive procedure, requiring 10-15 grams of thigh muscle. Moreover the tests are time-consuming and sometimes inconclusive. The concordance between tests is poor such that an individual may be labelled "at risk" by the criteria of one test and "not at risk" by the criteria of a second. Other individuals may be equivocal due to overlap in the values for "at risk" and "not at risk" groups. Control values and diagnostic "cut-off" points have to be established in each laboratory so that it is difficult to stablish n w units to test for MH susceptibility. Mor over, such an invasive technique is inappropriate for g neral population screening prior to

anaesth sia. Thus, the availability of a DNA based diagnostic test is of major significance and utility for det ction of individuals at risk for malignant hyperthermia.

Porcine halothane sensitivity represents an excellent animal model for malignant hyperthermia. The clinical crisis in pigs follows a very similar course to that in humans and crises may be similarly arrested or averted by prompt treatment with dantrolene sodium. In pigs, however, the syndrome may additionally be triggered by over-exercise and/or stress. Usually over-exercise is not a significant problem in the raising of pigs. However, stress is a problem as particularly experienced during shipping and prior to slaughter. The pig industry losses hundreds of thousands of dollars a year due to deaths or spoiled meat caused by pigs being susceptible to malignant hyperthermia.

While it has been described as a recessive condition in pigs, it is more likely to be a co-dominant condition since muscle biopsy studies [Britt, B. A., et al, "Malignant Hyperthermia - pattern of inheritance in Swine". In Aldrete J. A. eds., Second International Symposium on Malignant Hyperthermia. New York, Grune and Stratton, pp 195-211, (1978)] reveal that heterozygous pigs may be mildly affected and homozygous MH/MH pigs may be more seriously affected.

The gene responsible for halothane sensitivity (HAL) has been found to segregate in pigs with a number of other genetic markers including S (S Locus affecting expression of A-O red blood antigens), Phi (glucose phosphate isomerase), H (H locus encoding blood group antigens), Po2 (postalbumin-2) and PgD (6-phospho-gluconate dehydrogenase), [Archibald, A. L. and Imlah, P., Animal Blood Groups and Biochem. Genet. 16: 253-263, (1985)]. It is therefore assumed that these genes are linked on one pig chromosome. Since g netic linkage gr ups are often conserved throughout the animal

kingdom and since the human equivalents f three of these genetic markers (Phi, Po2 and H) have been found to map to human chromosome 19 [Shaw, D. and Eiberg, H., Report of the Committee for chromosomes 17, 18, and 19. Human Gene Mapping 9 (1987); Ninth International Workshop on Human Gene Mapping. Cytogenet. Cell Genet, vol 46, Nos. 1 - 4, (1987)], there was reason to suggest that the human gene for MH may also be in this gene cluster on chromosome 19. A possible further localization of MH to the long arm (q) of chromosome 19 was suggested by the fact that the H gene analog maps to 19q and the Phi gene analog (GPI) maps to band 19q12-19q13 on 19q (Shaw and Eiberg, supra).

In recent years it has been possible to track genetic disease genes in families using closely linked genetic markers. The most commonly used marker of a chromosome site is a restriction enzyme cleavage site that may be present (+) on the pair of chromosomes in some members of the population or absent (-) at the same site in the chromosome pair of other members of the population. Still other individuals will be heterozygous (+/-) having one chromosome of each type [Botstein, E. et al, Am. J. Hum. Genet. 32: 314-331, (1980)]. The (+) chromosome can be distinguished from the (-) chromosome by extracting DNA from the blood cells (or other cells) of the test individual, treating it with the restriction enzyme whose cleavage site is "polymorphic" (i.e. cleaves or doesn't cleave) and fractionating the DNA fragments by size. The DNA from a chromosome without the cleavage site gives a larger fragment than the DNA from a chromosome with the cleavage site, providing an assay for the presence or absence of the cleavage site. The term RFLP is an acronym for restriction fragment length polymorphism. An RFLP constitutes a genetic marker allowing the polymorphic chromosome site to be tracked in a family . Any genetic disease that tracks (segregates) in a family with the RFLP marker is considered to be

linked to the marker, that is it maps n ar to the mark r on the chrom some which th y share.

SUMMARY OF THE INVENTION

According to an aspect of the invention, substantially purified DNA sequence encoding for human ryanodine receptor and its functional equivalents is characterized by:

i) the DNA encoding for a protein having 5032 amino acids and a molecular weight of approxmately 563,000 daltons;

ii) the DNA having a length of approximately 15.3 kb; and

iii) isolated from chromosome 19 of humans.

According to another aspect of the process, substantially purified cDNA has the sequence encoding for the amino acid positions 1 to 5032 of Figure 2.

According to another aspect of the invention, a DNA probe includes a fragment of the DNA encoding for the human ryanodine receptor protein.

According to a further aspect of the invention, the use of the DNA probe in RFLP analysis determines if a subject is susceptible to malignant hyperthermia.

According to another aspect of the invention, substantially purified ryanodine receptor protein is free of any foreign human protein. The protein has a molecular weight of approximately 563,000 daltons and an amino acid sequence of Figure 2.

According to another aspect of the invention, substantially purified antibodies specific to a protein fragment of the human ryanodine receptor protein is disclosed. The antibodies may be either polyclonal antibodies or monoclonal antibodies. Such antibodies may be used in an amino acid assay to determine if a human or animal is susceptible to malignant hyperthermia.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodim nts of the invention are discussed with respect to the drawings, wherein;

Figure 1 is a schematic drawing sh wing the restriction endonuclease map and sequencing strategy of the human ryanodine receptor (HRR).

Figure 2 is the nucleotide sequence (upper row) and deduced amino acid sequence (lower row) of the human ryanodine receptor cDNA. Sequences underlined once correspond to peptide sequences determined from the purified receptor protein. Sequences underlined twice are potential phosphorylation sites.

Figure 3 is a Southern blot hybridization using human-rodent somatic cell hybrids to localize the HRR gene to human chromosome 19.

Figure 4 is a Southern blot hybridization using somatic cell hybrids derived from chromosome 19 reciprocal translocation, to further localize the ryanodine receptor gene on human chromosome 19.

Figure 5 is a Restriction Fragment Length Polymorphism (RFLP) analysis of two representative malignant hyperthermia families.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The sustained muscle contraction in MH suggests that the problem in this disease may be related to the release of calcium into the muscle cell cytoplasm from the sarcoplasmic reticulum (SR). The calcium release channel in muscle is a large protein that spans the gap between a membranous structure called the transverse tubule and the SR. Recently evidence has been accumulated to show that this protein is equivalent to the "ryanodine receptor" protein and researchers have speculated that a defect in the channel might be the basic defect in animals, including humans, with MH.

This invention shows that the ryanodine receptor gene is the gene that is defective in MH. This application discloses isolation of the gene that encodes the human ryanodine receptor protein, mapping of the human ryanodine receptor (HRR) gene to human chromosome 19, further mapping the MH gene to the long arm of

chromosome 19 and determination that RFLP markers within the HRR gene segregat 100% f the time with the MH ph notype. The HRR gen is beli ved to be the gene responsible for MH and DNA probes from the HRR gene constitute the basis for a definitive diagnostic test for MH.

The primary defect in MH is believed to be in calcium regulation. Myoplasmic calcium rises rapidly during onset of MH in MH susceptible pigs, and this is rapidly reversed by administration of dantrolene [Lopez, J. R., Allen, P., Alamo, L., Jones D., and Sreter F. Muscle Nerve, 11, 82-88, (1988)]. While the calcium-ATPase protein (Calcium pump) appears to function normally in both human and pig MH muscle, several studies indicated a defect in the calcium induced calcium release channel.

The resting level of calcium is elevated in the mycoplasm of MH muscle while other reports indicate that calcium induced calcium release is activated at a lower calcium threshold in heavy SR isolated from MH pigs than in heavy SR isolated from normal pigs and that this threshold is further lowered in the presence of halothane. Ohta, T, Endo, M, Nakano, T., Morohoshi, Y, Wanikawa K, Ohga, A, American Physiological Society C358-367, (1989) reported that muscle from MH sensitive (MHS) pigs has a significantly higher rate of calcium induced calcium release than normal. Mickelson et al (J. Biol. Chem. 264: 1715-1722, 1988) also showed an increased rate of calcium induced calcium release in MHS muscle.

The calcium release channel in muscle is a large protein that spans the gap between the transverse tubule and the SR. The channel is activated by calcium, caffeine, halothane, and micro-molar ryanodine, and inhibited by ruthenium red, tetracaine, calmodulin, high Mg^{2+} and mM ryanodine [Lai F. A. et al, Nature, 331: 315-319, (1988)]. The resting level f calcium is elevat d in the myoplasm of MH muscle (Lopez et al, supra), and

calcium-induced calcium release is activated at a low r calcium threshold in MH pigs than in normal pigs. This threshold is further lowered in the presence of halothane (Nelson, 1988). Ohta et al supra, and Mickelson et al. supra, both reported an increased rate of calcium induced calcium release in MHS muscle. The latter study indicated that the ryanodine receptor from MHS muscle has a higher affinity for ryanodine binding and requires higher concentrations of calcium to inhibit ryanodine binding.

Ryanodine causes contracture of skeletal muscle in vitro in both pig and human, similar to that induced by caffeine and/or halothane and reportedly discriminates more effectively between MH and normal muscle.

The rabbit ryanodine receptor has recently been cloned [Takeshima, H. et al., Nature, 339: 439-445, (1989)] independently of the work disclosed herein. The deduced amino acid sequence comprises 5037 amino acids. The predicted protein structure suggests that the calcium release channel, comprising four transmembrane domains and potential regulatory sequences, lies in the C-terminal portion of the molecule. The remainder of the protein is predicted to constitute the "foot" portion which spans the gap between the transverse tubule and the SR.

The C-terminal fifth of the predicted structure of the human ryanodine receptor contains from four to ten transmembrane segments which form the channel itself. Potential binding sites for calcium, calmodulin, ATP and other modulators of calcium channel function are also believed to be present in the molecule. Two of these transmembrane regions show limited homology to transmembrane regions of the nicotinic acetylcholine receptor (nAChR). Specific amino acid substitutions which alter the charge distribution in the Torpedo nAChR have been shown to alter channel conductance [Imota, K. et al Nature, 335: 645-648, (1988)]. By analogy, amino

acid substitutions in th h mol gous transmembrane segments of the human ryanodine receptor could alt r rates of calcium release and account for the MH phenotype.

The initial rabbit cDNA clones were obtained by screening a λgt11 expression cDNA library with affinity purified polyclonal antibody directed against epitopes on the purified rabbit ryanodine receptor protein as described in [Zorzato, F., et al, Biochem. J. 261: 863-870, (1989)]. The protein sequence is published in Takeshima et al (supra) and hence readily available to the public. Two initial rabbit cDNA clones were isolated from this library. Restriction endonuclease fragments from these clones were then used as probes to isolate longer cDNA clones from a neonatal rabbit skeletal muscle cDNA library. This library is freely available and is described in MacLennan, D. H., et al, Nature 316: 696-700 (1985). All the libraries used in this application are freely available. It is understood by those skilled in the art, however, that any library prepared from skeletal muscle according to established methods should be representative of the RNA species present in the tissue, and can therefore potentially be the source of a ryanodine receptor clone.

Further extension of the clones was achieved by the use of a primer extension library using neonatal rabbit muscle poly A+ RNA.

The rabbit ryanodine receptor cDNA probes were used to isolate the HRR cDNA on the basis of cross-species conservation. A series of six linear clones were isolated from a human skeletal muscle cDNA library in λgt10. These clones are presented in Figure 1, labelled as cDNA 1 through 6. cDNA clone 7 in Figure 1 was isolated from a primer extension library constructed from human skeletal muscle mRNA.

Genomic DNA spanning the non-overlapping cDNA fragments shown in Figure 1 was isolated from a

chr m s me 19 specific library, designati n Lawrence Livermor LL19NL01 Human Chr mosome 19 Library in Charon 4A by the American Type Culture Collection, Rockville, MD.

Figure 1 presents the restriction map and sequencing strategy for the human ryanodine receptor cDNA. The first line shows the size in kb of the full length cDNA. The second shows a partial restriction endonuclease map of the 15.3 kb cDNA which encodes the human ryanodine receptor protein. The third and fourth lines define the 6 cDNA clones (1-6) that were isolated from the first cDNA library and the single clone (7) that was isolated from a primer extended human cDNA library. Arrows inside the clones indicate that they were all sequenced in two directions. The fifth line designates the regions where genomic DNAs were sequenced to obtain overlapping sequences between the various clones. HRR probes 1,2,3,4,5, and 6 described in this application, correspond to cDNA clones 1,3,4,5,6 and 7, respectively.

Figure 2 presents the nucleotide and deduced amino acid sequences of the cDNA encoding the human ryanodine receptor. The nucleotide are numbered positively beginning at the first residue of the initiator methionine codon. The nucleotide comprising the 5′ non translated region are numbered negatively in the 3′ to 5′ orientation. The deduced amino acid sequence of the open reading frame is numbered from 1 to 5032. Peptide sequences determined from the purified receptor are underlined once. The potential phosphorylation sites are underlined twice. The 3′ untranslated region, beginning after the TGA termination codon, is 142 bp long. A canonical AAAATAAA polyadenylation signal [Proudfoot, N. J. and Brownlee, G. G., Nature 263: 211-214, (1976)] is found 19 bases upstream of the polyadenylation site and this is followed closely by the TG-rich sequence TCTGTCGTACG, characteristic of sequences betw n the polyadenylation signal and the polyadenylation site

[McLauchlan, J. et al., Nucleic Acids Res. 13: 1347-1368, (1985)]. The initiator methionine is 15096 bp upstream of the termination codon. The initiator methionine codon is present in the longer sequence ACATCATGG which closely resembles the consensus initiation sequence, CCA(G)CCATGG [Kozak, M, Nature 308: 241-246, (1984)]. The human cDNA sequence of Figure 2 encodes a protein of 5032 amino acids with a predicted molecular weight of 563,584. It is understood that the term substantially pure as used herein means that the isolated and purified DNA or protein is free of any foreign animal DNA or protein. It is also understood that, with reference to disclosed and claimed DNA or protein sequences, various functional equivalents exist which are due to substitutions in variable regions of the DNA sequence or protein which does not affect the essential function of the DNA sequence or protein sequence.

Proof that the cDNA coded for the ryanodine receptor gene was provided by several lines of evidence. The fusion protein expressed by the clones reacted with a second antibody raised against the purified 30S ryanodine receptor [Meissner, G. Rousseau, E. and Lai, F. A. J. Biol. Chem. 264: 1715-1722, (1989)]. As further evidence, both rabbit and human probes from the coding region of the cDNA hybridized to a message of approximately 15 kb in rabbit muscle mRNA . Tryptic peptides prepared from the rabbit ryanodine receptor were sequenced. The primer extension and DNA sequence analysis of the cDNA clones revealed four deduced amino acid sequences corresponding to the sequences of the tryptic peptides derived from the purified ryanodine receptor protein. These sequences are underlined in Figure 2. Further evidence that the clones encoded the ryanodine receptor is provided by noting that the deduced amino acid sequence would give rise to a protein with several transmembrane passages only in the carboxyl-terminal fifth of the molecule and that the bulk

of the protein was hydrophilic. Such a predicted protein would match very well with the structure of the ryanodine receptor in which the bulk of the protein is cytoplasmic and only a small segment is transmembrane [Wagenknecht, T. et al., Nature 338: 167-170, (1989)].

Localization of the ryanodine receptor gene on human chromosome 19 was determined using a panel of human-rodent somatic cell hybrids [MacLennan, D. H. et al., Somat. Cell and Mol. Genet. 13: 341-346, (1987)]. Figure 3 shows filter hybridization analysis of the human ryanodine receptor gene. DNAs are from human (lane 1), mouse (lane 2) and mouse-human hybrids (lanes 3 and 4) (MacLennan et al supra). DNAs were digested with EcoR1 and Southern blot analysis was performed with the probe pHRR-XH-1, a 1001 bp Xho1 - Hind III fragment from the human ryanodine receptor cDNA, consisting of bases 8550 to 9550 in the human cDNA sequence (Figure 2). The hybrid in lane 3 contained human chromosome 19; the hybrid in lane 4 did not. The presence or absence of chromosome 19 in the 13 somatic cell hybrids used in this study was confirmed both cytogenetically and with DNA probes from chromosome 19. The 15 kb EcoR1 band in human genomic DNA hybridizes to the probe pHRR-XH-1. Cross hybridization to mouse bands at 2.4, 2.1 and 0.5 kb was also observed. Analysis of a series of 13 somatic cell hybrids revealed 100% concordance for the presence of chromosome 19 and the presence of a 15 kb EcoR1 restriction fragment which hybridized to the human ryanodine receptor probe pHRR-XH-1 as shown in Figure 3. As shown in Table 1, for all other chromosomes, including X and Y chromosomes, discordance ranged from 23% to 77%.

TABLE 1

Chromosome Mapping of Human Ryanodine Receptor Gene

Chromosome No.	Discordant	Discordancy (%)
1	3	23
2	4	31
3	6	46
4	9	69
5	8	62
6	5	38
7	5	42
8	5	38
9	5	38
10	5	38
11	5	38
12	5	38
13	9	69
14	6	46
15	6	46
16	4	31
17	7	54
18	5	38
19	0	0
20	7	54
21	6	46
22	10	77
X	7	54
Y	6	46

Chromosome 19 regional sublocalization of the gene identified by the probe pHRR-XH-1 was accomplished using a panel of somatic cell hybrids that were derived from a variety of reciprocal translocations involving human chromosome 19. The construction and analysis of these hybrids have been described by Brook, I. D. et al., Hum. Genet. 68: 282-285, (1984), Bufton, L. et al., Am. J. Hum. Genet. 38: 447-460, (1986), Korneluk, R. G. et al.,

Genomics in press, (1989); Lusis A. J. et al., Proc. Nat. Acad. Sci. U.S.A. 83: 3929-3933, (1986); Mohandas T. t al., Proc. Nat. Acad. Sci. U.S.A. 77: 6759-6763, (1980). Figure 4 shows genomic DNA extracted from hybrid and control cells, digested to completion with Eco R1 and separated by electrophoresis for subsequent Southern blot analysis. The autoradiograph depicts the hybridization pattern of the probe pHRR-XH-1 to DNA extracted from (1) Mouse A9 control; (2) Hamster E36 control; (3) G24A9 (retains 19p13-->19qter); (4) G24B2TG (back selected for the loss of 19p13 -->19qter); (5) G35E4 (contains 19 pter --> 19q13.3); (6) B-9 (has 19q13.2 --> 19qter); (7) GM89A99C7B (retains 19q13.3 --> 19qter); (8) CF104-19/6 (has 19 cen --> qter); (9) G89E5 (contains a normal human X chromosome only); (10) normal human male; (11) normal human female. The EcoR1 fragment from human chromosome 19 which hybridizes with probe pHRR-XH-1 is approximately 15 kb in size. Cross-hybridization of the probe pHRR-XH-1 to hamster DNA sequences is seen at 6.0 kb and to mouse EcoR1 fragments at 2.4 and 2.1 kb. Figure 4 illustrates that the HRR probe hybridizes to genomic digests of human DNA from hybrids that retain the proximal 19q region; p13 --> qter (lane 3), pter --> q13.3 (lane 5) and cen --> qter (lane 8). By contrast, hybrids that contain the distal 19q regions q13.2 --> qter (lane 6) and q13.3 --> qter (lane 7) did not show binding to the probe pHRR-XH-1. These results sublocalize the HRR probe to the proximal 19q region in the interval cen --> q13.2.

To demonstrate that the HRR gene is the gene that, when mutated, is the cause of MH, RFLP markers that are part of the HRR gene have been found to segregate with the MH gene (and therefore the MH phenotype) in families. To show this, blood samples from members of several MH families w re obtained and subjected to RFLP analysis. The RFLPs examined included s veral previously r ported genetic markers that had been mapped to chromos me 19.

More importantly several new RFLPs are described that are recognized by cDNA probes from the HRR gene, ther by establishing the means to test the HRR gene directly for co-segregation with the MH phenotype.

RFLPs in the HRR gene were identified by using various cDNA clones from the HRR gene as probes to detect hybridizing sequences on Southern blots of DNA from a set of normal individuals. Samples of the test DNA were cleaved with each of several restriction enzymes, the fragments were size fractionated by electrophoresis in agarose gels and blotted to a nylon membrane. Denatured DNA on the membrane was hybridized by standard procedures with 32P labelled HRR cDNA probes, and the position of hybridizing sequences was determined by standard autoradiographic procedures. Any probe / enzyme combination that revealed a variation in fragment size among the tested individuals defined a new RFLP.

In total, eleven RFLPs were defined in the HRR gene. Their properties are summarized in Table 2. For each RFLP found the HRR cDNA probe is listed in column 1, the restriction enzyme with the polymorphic site is listed in column 2, the variable fragment sizes (alleles) are provided in column 3, constant size fragments in column 4, and the frequencies of the alleles are provided in column 5.

TABLE 2

Human Ryanodine Receptor Probe Information

Probe	Polymorphic Enzyme Enzyme	Polymorphic Fragment Sizes (Alleles)	Constant Bands	Allele Frequencies
HRR1-600	Ban I	6.0/13/0		.22/.78
HRR3-1000	Hind III	19.0/22.		.04/.96
	Pvu II*	6.5/4.4/1.9		.25/.75
	Bam HI*	19.0/14.0,5.0		.75/.25
HRR3-1200	Pvu II	1.9,3.9/5.8		.11/.89
HRR4-2400	Taq I	1.8/1.1,0.7	3.5	.20/.80
	Bcl I	11.5/8.4,2.9	4.6,3.0	.78/.22
	EcoRV	28.0/2.5	11.0	
HRR5-3800	Eco RI	24.0/16.0,9.0		.14/.86
	Taq I	1.9/1.6,0.3		
	Msp I	1.8/2.2		

*Pvu and Bam HI polymorphisms are in total linkage disequilibrium with each other, and were treated as a single genetic marker.

These probes can be defined more particularly by reference to the nucleotide sequence of Figure 2. HRR 1-1600 is an approximately 1.6 kb fragment spanning nucleotides 13602 to 15243 shown in Figure 2. HRR 2-2000 is an approximately 2 kb fragment spanning nucleotides 11613 to 13607 in Figure 2. HRR 3-3100 is an approximately 3.1 kb fragment spanning nucleotides 8515 - 11618 shown in Figure 2. HRR 3-1000 is a subfragment of HRR 3-3100, of approximately 1 kb, spanning nucleotides 8515 - 9554 shown in Figure 2. HRR 3-1200 is also a subfragment of HRR 3-3100, of approximately 1.2 kb, spanning nucleotides 9549 - 10851 shown in Figure 2. HRR 4-2400 is an approximately 2.4 kb fragment spanning nucleotides 6125 - 8493 shown in Figure 2. HRR 5-3800 is an approximately 3.8 kb fragment spanning nucleotides 2391 - 6125 shown in Figure 2. In addition, HRR 7A (nucleotides 186 - 2396) and 7B (nucleotides -104 - 548) have been tested, but have not revealed any RFLP markers with the restriction enzymes tested.

With this set of HRR gene markers, it was possible to test for segregation of the markers with the MH phenotype in MH families. Families selected for study had been under investigation for many years at the Toronto General Hospital. Families chosen were those with a potential for at least two informative offspring, (ie. ones in which the MH gene has been inherited from an affected parent who is heterozygous for both the MH gene [one normal and one mutant allele] and the genetic marker at the HRR gene [one allele of each type]). Family members were classified as affected if they had either an MH reaction or muscle biopsy results which were unequivocally affected. In some cases the status of the second parent had to be inferred as only one parent had been biopsied. Muscle biopsies were performed according to the North American Malignant Hyperthermia protocol (Britt, 1988) as described below. Biopsy test results were considered to be unequivocal if at least two

parameters wer consistent, not being intermediate and/or ambiguous.

Three muscle biopsy drug tests were performed on separate muscle strips - halothane, caffeine, and caffeine plus halothane. These tests are all based on the different contracture responsiveness of normal and Malignant Hyperthermia affected muscle (Table 3).

TABLE 3

Muscle Biopsy Test Criteria (age > 10 years)

	Normal	Affected
Halothane 1%	< 0.2 g	> 0.3 g
3%	< 0.7 g	> 0.8 g
Caffeine	> 4.0 mM	< 4.0 mM
Caff. + Hal.	> 0.5 mM	< 0.35 mM

Caffeine - added cumulativele (1,2,4,8,16,32 mM)
measure contracture - baseline to plateau
Caff + Hal. - caffeine added muculatively (0.25,0.5,1,2, 4,8,16,32) after 10 min in 1% halothane

Muscle biopsies were taken from affected individuals and their first degree relatives with a requirement for a lean body mass of 20 kilograms. At least two to three months was left between an MH reaction and the diagnostic biopsy. Surgery was performed using nitrous oxide, Innovar and midazolam. No dantrolene was administered prior to surgery as this can alter test results. The muscles chosen for biopsy were the vastus lateralis, rectis abdominis, or gracilis. These three muscles all react similarly in the caffeine halothane contracture test.

Good twitch viability in each specimen was demonstrated prior to testing. A 20 minute period was allowed in order to establish stable twitch heights and baselines prior to addition of drugs. 1% halothane contracture tests were performed on all individuals. 3% halothane testing was done on some individuals. In each

case exp sure time was 10 minut s. The amplitude of the contractures was measured and a contracture of greater than 0.3 g at 1% and 0.7 g at 3% was defined as abnormal. For the caffeine test, caffeine is added incrementally at 4 minute intervals from 0.5 mM doubling to 32 mM. Contractures in response to each dose were measured four minutes after each addition of drug. The dose of caffeine required to raise the resting tension by 1 g (Caffeine Specific Concentration) was calculated. Values greater than 4mM were considered normal. In the caffeine plus halothane test, muscle strips were equilibrated for 15 minutes with 1% halothane. Caffeine was then added incrementally from 0.25 mM doubling to 32 mM and the Caffeine Specific Concentration in the presence of Halothane was calculated similarly. Values greater than 0.5 mM were considered to be normal. Individuals with values less than 0.35 mM were considered to be affected. Table 3 lists the test criteria that were used to determine whether a result was positive or negative. In general the caffeine plus halothane test was taken as the most definitive and in most instances the caffeine test was in agreement with this. The halothane test (with 1% halothane) tended to give false negatives and it was ignored if the two other tests were positive. To avoid possible confusion, families with discrepant results or families that did not fit the standard criteria for autosomal dominant inheritance were not used for RFLP analysis. On this basis 19 families were chosen for study.

Two examples of typical families are presented in Figures 5a and 5b. In Figure 5a, data are presented for family 5 . The mother has had a reaction (R) and also scores positive for the caffeine plus halothane test (K). Her husband is presumed to be normal. Three children are affected according to the biopsy results. Segregation was examined for three HRR gene RFLPs, two of them recognized by HRR3 and one by HRR4. There is one

constant band (C). The three pairs of polymorphic fragments are 22 and 19 kb (HRR3 with HindIII - table 1), 11.5 and 8.4 kb (HRR4 with BclI - a 2.9 kb fragment that goes with the 8.4 kb fragment is not shown - table 1) and 6.5 and 4.4 kb (HRR3 with PvuII - a 1.9 kb fragment that goes with the 4.4 kb fragment is not shown- table 1). For each of the RFLP markers the father is homozygous for one allele and the mother is heterozygous for the two alleles. In the case of the first RFLP, for example, the father has 22/22 and the mother has 22/19. All three affected children received the 22 kb marker from both parents. Thus all three received the 22 kb allele from mother along with the MH gene they also received from her. While this could occur by chance it is consistent with the MH gene and the HRR gene segregating together due to close linkage or identity of the two genes. The same reasoning applies to the other two RFLPs that were informative in this family.

Figure 5b shows Family 12, in which the father and two offspring are affected. Two RFLPs were examined, one with a constant size fragment (C) and alleles of 6.5 and 4.4 kb (as in Figure5a) and one detected with a probe from the ApoCII gene (Shaw and Eiberg, supra) that maps close to the HRR gene on chromosome 19. The latter detects alleles of 3.8 and 3.5 kb. For the HRR3 RFLP father is heterozygous 6.5/4.4 and mother is homozygous 4.4/4.4. All four offspring must have received a 4.4 kb allele from mother. The two normal girls received 4.4 from father while the two affected individuals received the 6.5 kb allele from him. This could have occurred by chance but it is also consistent with the 6.5 kb allele segregating with the mutant MH gene on one chromosome 19 and the 4.4 kb allele segregating with the normal MH gene on the other chromosome 19. A similar result is obtained for the ApoCII g netic mark r with the two normal girls receiving the opposite ApoCII alleles from their affected siblings. This is consistent with linkage of the ApoCII

g n with the MH gene but could also b a chance occurrence.

These two representative families illustrate that the genetic test for linkage of chromosome markers with the MH gene is a statistical one. In any family the segregation pattern could always occur by chance or by co-segregation of the two markers with the disease due to true genetic linkage. To demonstrate that linkage is more likely than chance occurrence requires that several families be examined and that a statistical analysis be performed. Table 4 summarizes the data from 19 families examined. Eight families gave informative results with one or more of the HRR gene probes, with results obtained on 19 offspring of affected parents. In every family the affected children all received the same HRR marker and the unaffected ones all received the alternate form. The probability of this occurring by chance is small. Standard linkage analysis was performed to determine the odds of this result being due to linkage rather than chance. The results are as shown in Table 4.

TABLE 4

MH GENE AND HRR GENE LINKAGE

Linkage Dist. (CM)	LOD Score
0.00	3.31
0.01	3.22
0.02	3.15
0.05	2.91
0.10	2.51
0.20	1.71
0.40	0.30

The tabulated values are the log of the odds (to base 10) otherwise known as the LOD score. Performing this calculation for different possible presumed distances between the HRR gene and the MH gene demonstrates that the highest odds are attained at a presumed distance of zero. This suggests that the most likely interpretation is that the two genes are the same. Since three is the log of 1000, a LOD score of greater than three indicates that the odds in favor of linkage versus chance are greater than 1000 to 1. It is reasonable to conclude that the HRR gene is in fact the gene that, when mutated, is the cause of MH. This conclusion is strengthened by the fact that among eight additional families who are not informative for the HRR gene probes, but were informative for genetic markers that flank the HRR gene on chromosome 19, only three recombination events were detected between the MH gene and the flanking marker. This also provides definitive evidence that the MH gene maps to the same region of the chromosome as the HRR gene.

RFLP markers detected by HRR cDNA clones segregated 100% of the time with the MH phenotype in families informative with these probes. Given that all the relevant family members are available for DNA testing, it can be possible to determine the MH status of family members who have not had an MH reaction or a muscle

biopsy. Both the affect d individual and his r her parents or siblings whose MH status is known must be available. For example, if a parent and child are affected, determining the status of the other children in the family would be possible if both parents and the affected child are available for DNA testing. In another example, it is possible to diagnose the first offspring in a family where both a parent and grandparent are known to be affected. In this case, it would be necessary to have both parents and grandparents as well as the child to study. At present the test requires that one of the eleven markers previously defined be informative in a family for the diagnosis to be made. Eight of the nineteen families used to establish the LOD score fit these criteria.

Markers which flank the HRR gene may also be used to track the disease but the risk of a recombination event (which uncouples the markers from the disease phenotype) will increase with distance from the MH gene and result in the misdiagnosis rate of a few percent. New markers can be developed by screening genomic clones detected by the HRR cDNA for multiple copies of dinucleotide repeats. Such markers are analysed by the polymerase chain reaction (PCR) and have multiple alleles in the population [Weber, J. L. and May, P. E. Am. J. Hum. Genet. 44: 338-396, (1989)]. These can provide intragenic probes informative in a greater proportion of families and thus avoid the need for flanking markers with their associated risk of recombination with the disease phenotype.

Analysis of current linkage data does not indicate the existence of a common haplotype(s) associated with human MH which indicates that multiple mutations of the MH gene may be found. In order to develop a test for general population screening, it will be necessary to find and charact rize each mutation.

Kn wledge of the n rmal HRR cDNA sequence disclos d herein allows amplification f segment cDNA copies of the normal and mutated genes using the polym rase chain reaction. Mutations will be apparent as sequence discrepancies (with respect to the cloned DNA sequence) in the heterozygous state (for direct sequencing) or in the hemizygous state (for cloned sequence). By using cDNA, RNA processing mutations will be detected in addition to base substitutions, deletions, duplications, or insertions.

Once a mutation is defined, oligonucleotides, derived from the normal sequence may be synthesized which flank the mutation and can be used to amplify both normal and mutant alleles. Short oligonucleotides containing either the mutated region of the HRR gene or the corresponding normal site of the HRR gene should then be synthesized and used as probes, in turn, to test blots of amplified DNA containing the region of interest. The mutant sequence oligonucleotide should hybridize only to DNA from affected individuals, whereas the normal sequence oligonucleotide should hybridize to DNA from both affected and unaffected individuals. It should also be possible to design an allele specific oligonucleotide based on the sequence of a mutation(s), which in conjunction with a normal HRR sequence oligonucleotide will, in a PCR reaction, generate product only in affected individuals.

To confirm that potential discrepancies represent a mutation, rather than a polymorphism, each such sequencing discrepancy must be examined in both normal and affected individuals by means of restriction enzyme analysis (if the mutation creates or destroys a site), by allele specific amplification, by differential oligonucleotide hybridization, chemical cleavage [Cotton et al, Proc. Nat. Acad. Sci.U. S. A., 85: 4397-4401, (1985)], denaturing gradient gel electrophor sis [Myers and Maniatis, Cold Spring Harbour Symp. Quant. Biol. 51:

275-284, (1986)], RNas protecti n [Myers, R. M., Larin, J., and T. Maniatis, Science 230: 1242, (1985)], or by DNA sequencing, as appropriate. A discr pancy which would alter the amino acid constitution of the HRR protein and which occurs in several affected individuals, but never in normals, would almost certainly represent a mutation. Alternative explanations such as segregation of a polymorphic marker closely linked to the mutation are unlikely, given that common MH haplotypes have not been observed. Once mutations are confirmed, further diagnostic tests may be developed.

DNA sequencing of genomic clones can be performed to determine intron-exon boundaries for the HRR gene. As these are established, it becomes possible to amplify from DNA rather than cDNA, enabling investigation on patients for whom muscle biopsy samples are not available and to expand the study to include additional patients as each mutation is defined. Such an application would also allow one to detect mutations in introns or at intron/exon borders that affect splicing of the message and lead to aberrant protein which would only be inferred from a cDNA based analysis.

The ease of developing a general diagnostic test for MH is dependent on the number of mutations in the gene that cause the MH phenotype. As each mutation is traced a test may be developed which may be informative in all individuals in the family in which it was found and for others which share the mutation. Common mutations may be screened for amongst patients presenting for diagnostic biopsy and once a mutation or mutations comprising a cost and time effective proportion of MH individuals are described, a general population test can be developed.

The type of genetic analysis using RFLPs in humans herein described can also be applied to pigs. Cross species hybridization allows the direct detection of pig RFLPs with the human cDNA. RFLP's det cted by the porcine GPI cDNA and GPI gene, closely linked to Hal (MH)

have been succ ssfully used in this type f analysis [Davi s, W. et al. Animal Genetics 19: 203-212, (1989)].

Hal has been shown to be the gene responsible for both porcine stress syndrome (PSS) and pale, soft exudative pork (PSEP) which result in unfavorable meat quality due to myolactosis in the immediate one to two hours post-slaughter. This represents a considerable economic problem in North America and Europe, with estimated annual losses of 300 million dollars in the United States alone [Harrision, G. G., "Porcine Malignant Hyperthermia, the Saga of the Hot Pig", in Britt, B. A., Ed. Malignant Hyperthermia, Boston, Martinus Nijhoff, 103-136, (1987)]. A DNA diagnostic test would provide a cheap and reliable means of determining MH status particularly in breeding programs and should assist in the design of breeding programs which aim to lose the Hal trait while maintaining beneficial characteristics. The RFLP analysis in pigs would be able to eliminate the Hal gene from several strains resulting in considerable saving in the industry.

The human cDNA clones as disclosed herein may be used to isolate pig cDNA clones from both homozygous normal and homozygous affected pigs. The sequence of the two genes can then be compared to determine the mutation in each strain. Once these are identified,, tests similar to those outlined above can be developed to test the authenticity of and/or diagnose the porcine mutations. Knowledge of these mutations will allow for the identification of affected and heterozygous pigs without resorting to RFLP analysis and will extend the utility of the detection method to include the identification of affected pigs in herds in which the gene frequency is rare.

It is also feasible to develop polyclonal or monoclonal antibodies against specific peptide sequences that can be used to detect differences in normal and mutant ryanodine receptor proteins. It is likely that MH

individuals (either human or porcine) have a section of the pr tein sequence either missing or altered. It is possible to use a specific segment of peptide sequence to make fusion protein antigens and or synthetic peptide antigens and raise antibodies to the specific segment. Thus, antibodies raised to the altered sequence (Mutant MH) should show positive immunostaining on muscle sections in human MH individuals, MH pigs, and heterozygous pigs, as they will react with antigen in the muscle. No staining should be observed in normal human or porcine individuals. Heterozygous pigs should be distinguishable from honozygous affected pigs by the use of antibody raised to the normal peptide. Similarly, these reactions could be observed with the more sensitive technique of Western blotting of extracts of muscle protein.

The DNA sequence disclosed herein can be manipulated in order to achieve expression and production of large quantitites of the protein for functional analysis and antibody production. Partial or full length cDNA sequences, which encode for HRR may be ligated to bacterial expression vectors (for example, pRIT, pGEX, or pATH). The DNA can also be transferred from its existing context to other cloning vehicles, such as other plasmids, bacteriophages, cosmids, animal virus, yeast artificial chromosomes, somatic cells, and other simple or complex organisms, such as bacteria, fungi, invertebrates, or plants. The cDNA sequence (or portions thereof) or a mini gene can be introduced into eukaryotic expression vectors by standard techniques. The expression vectors can also be introduced into recipient cells by conventional techniques, including transfection, calcium or strontium phosphate precipitation, electroporation, microinjection, protoplast fusion, or virus vectors.

Antibodies to various epitopes of the HRR protein can be raised to provide extensive information on the

characteristics of the prot in and other valuable inf rmation which can be used in immunoassays. The antibodies may be raised to fusion proteins containing defined portions of the HRR polypeptide or may be raised to protein fragments which are prepared by chemical synthesis. As already noted, fusion proteins may be developed by expression of vectors in suitable hosts. As to the chemical synthesis of the proteins, this is well within the skill of those knowledgeable in the art.

With the purified protein, which may be a developed by affinity chromatography, the protein fragments are coupled to a suitable carrier protein and injected into rabbits. Sera from the rabbits immunized with the protein fragments is screened in accordance with standard techniques to raise polyclonal antibodies. In accordance with well understood techniques, monoclonal antibodies may also be raised in accordance with the methodology of Kohlar and Millstein [Nature, 256:495 (1975)]. Mice are immunized with the selected protein as conjugated with suitable carrier. Hybridomas are developed and screened to select the desired monoclonal antibody. The antibodies, either poly or mono, are used in diagnostic determinations to detect the presence of certain epitopes of protein.

DNA sequences may also be subjected to site directed mutagenesis for comparitive function expression studies. If the nature of the human and pig mutations are very different, as is suggested by the different modes of inheritance of MH in humans (dominant) and pig (co-dominant), it may be desirable to create animal models which more closely resemble each human mutation. This could be achieved by site directed mutagenesis in conjunction with homologous recombination in embryonic stem cells, or by use of retrovirus vectors to create transgenic animals.

EXAMPLE 1

The Ca2+ release channel complex of rabbit skeletal muscle was isolated from heavy sarcoplasmic r ticulum membranes enriched in [3H]ryanodine binding and 45Ca2+ release activity by solubilization in CHAPS, followed by density gradient centrifugation through 5-20% sucrose (Lai et al., supra). The [3H]ryanodine receptor peak was collected, concentrated and recentrifuged. SDS polyacrylamide gel analysis of the pooled [3H]ryanodine receptor peak on a linear 5-12% polyacrylamide gradient gel revealed a single major high molecular weight band with an apparent relative molecular mass of approximately 400,000 daltons (Lai et al., supra). Therefore, in accordance with this technique, the ryanodine receptor protein was isolated and purified.

Hydrophilic tryptic peptides from the purified ryanodine receptor were separated by reverse phase-HPLC and subjected to automated NH2-terminal analysis by Edman degradation in an Applied Biosystems 470 gas phase sequenator with an on-line HPLC system for phenylthiohydantoin derivative analysis.

EXAMPLE 2.

The λgt11 cDNA expression library, constructed from poly(A)+RNA from rabbit fast-twitch psoas muscle as described in Ellis, S. B. et al., Science 241: 1661-1664, (1988). It is understood by those skilled in the art that any library prepared from skeletal muscle according to established methods should be representative of the RNA species present in the tissue, and can therefore be the source of the clone to be isolated. The library was screened with an affinity-purified polyclonal antibody (Zorzato et al., supra) specific for the Ca2+ release channel. Screening of the library was carried out by the method of Young and Davis, Proc. Nat. Acad. Sci. U.S.A. 80: 1194-1198, (1983).

The screening of 3 x 10^6 recombinant clones led to the is lation of two cDNA clones in the region defined by

nucl tide 14280-14629 and 13434-13758 in Figure 2. Analysis of the s quenc s of these clon s showed that both were rearranged when compared to the linear sequence of the human cDNA. Accordingly, restriction endonuclease fragments from these isolated cDNA clones were used as probes to isolate longer, unrearranged cDNA clones from the neonatal rabbit skeletal muscle cDNA library previously described by MacLennan et al., supra. The longest clone obtained was 6.8 kb and it is defined by nucleotide 8615-15241 in Figure 2. This clone was subcloned into the Bluescript® (Stratagene) plasmid vector and sequenced.

The clone was extended by construction of a primer extension library using 100 ug of neonatal rabbit muscle poly(A)+ RNA. The primer site was defined with 0.25 nmol of an 18mer oligonucleotide complementary to the rabbit equivalent of nucleotides 9118 to 9135 in Figure 2. Primer extension was carried out using the Bethesda Research Laboratories cDNA synthesis kit. In vitro packaging was performed with 1-2 ul aliquots of each ligation mixture using the Gigapack Gold packaging extract. Subsequent screening was carried out with the unamplified library. In the first primer extension, the cDNA was extended up to nucleotide 4527. In the second extension, an 18mer oligonucleotide complementary to the rabbit equivalent of residues 4892 to 4909 in Figure 2 was used to extend the cDNA to nucleotide 3231. In the third and final primer extension, an 18mer oligonucleotide complementary to the rabbit equivalent of residues 3499 to 3516 in Figure 2 was used to extend the sequence into the 5′ untranslated region of the mRNA.

EXAMPLE 3

Rabbit ryanodine receptor cDNA probes were used to screen a human skeletal muscle cDNA library in λgt10, described in Koenig, M. et al., Cell, 50: 509 - 517, (1987). In the first screen, over 30 clones w re isolated but only one, of about 2000 bp (clone 2 in Figure 1), had

an internal EcoR1 r stricti n site. All others terminated at an EcoR1 restriction site 1641 bp upstream of the poly(A) site (Figure 2), suggesting that the cDNA used to make the library was undermethylated, allowing the full length cDNA to be cleaved at EcoR1 sites prior to its ligation into the λgt10 vector. Accordingly, it was necessary to isolate rabbit cDNA clones first and then to use them as probes to identify and isolate new human cDNA clones. Eventually, a series of 6 linear cDNA clones were isolated from the library using rabbit cDNA probes (Figure 1). As a further complication in the isolation of human cDNA clones, an adenine rich region between residues 8501 and 8512 in Figure 2 acted as a second priming site for cDNA synthesis. While this led to the synthesis of an enhanced number of clones upstream of this site, it also terminated transcription. Thus clones 4 and 5 in Figure 1 were separated, not by an EcoR1 cleavage site, but by an actual gap in the cDNA. Clone 6 was the last cDNA clone isolated from the library and it represented the 5′ end of cDNAs primed at the internal Poly(A) site. The final clone, clone 7, was obtained from a primer extension library constructed from human skeletal muscle mRNA using the protocols that were used for primer extension of rabbit skeletal muscle mRNA. In this case the primer was a 17mer oligonucleotide complementary to residues 2620 to 2636 in Figure 2. Genomic DNA encoding sequences overlapping the various EcoR1 restriction sites and the gap in the cDNA introduced by the second primer initiating site were isolated from a chromosome 19-specific library under the designation Lawrence Livermore LL19NL01 Human Chromosome 19 library in Charon 4A by the American Type Culture Collection, Rockville, MD.

EXAMPLE 4

Muscle was clamped, carefully dissected and transported to the laboratory in carbogenated Krebs Ringer solution maintained at 22°C and pH 7.4. Also in

carb genated Krebs Ringer soluti n, biopsies w re trimmed fr e of fat and divided into strips of one to five mm by 10 to 20 mm of approximately 100 to 300 mg. Only intact muscle strips were used. Muscle strips were secured at one end by a silk suture to a plastic electrode frame and placed in a 40 ml bathing chamber containing Krebs Ringer solution at 37°C and pH 7.4 and aerated with 95% O_2, 5% CO_2 at 20 ml per minute. The muscle strip was then secured at the other end to a force displacement transducer which was coupled to a polygraph used to record the muscle tension.

Although preferred embodiments of the invention have been described herein in detail, it will be understood by those skilled in the art that variations may be made thereto without departing from the spirit of the invention or the scope of the appended claims.

CLAIMS

1. Substantially purified DNA sequence encoding for human ryanodine receptor, said DNA sequence being characterized by:

i) said DNA encoding for a protein having 5032 amino acids and a molecular weight of approximately 563,000 daltons;

ii) said DNA having length of approximately 15.3 kb; and

iii) isolated from chromosome 19 of humans.

2. DNA of claim 1 wherein said DNA has a restriction map of Figure 1.

3. Substantially purified cDNA having the sequence encoding for amino acid positions 1 to 5032 of Figure 2.

4. A DNA probe which includes a fragment of said DNA of claim 1, 2, or 3.

5. A DNA probe of claim 4 wherein a suitable label is attached to said DNA fragment to indicate hybridization in a DNA probe assay.

6. A DNA probe which includes one or more DNA sequences selected from the group of DNA sequences consisting of:

i) HRR-1 of approximately 1.6 kb spanning nucleotides 13602 to 15243 of Figure 2;

ii) HRR-2 of approximately 2 kb spanning nucleotides 11613 to 13607 of Figure 2;

iii) HRR-3 of approximately 3.1 kb spanning nucleotides 8515 to 11618 of Figure 2;

iv) HRR-3-1000 of approximately 1 kb spanning nucleotides 8515 to 9554 of Figure 2;

v) HRR-3-1200 of approximately 1.2 kb spanning nucleotides 9549 to 10851 of Figure 2;

vi) HRR-4 of approximat ly 2.4 kb spanning nucleotides 6125 to 8493 of Figure 2;
vii) HRR-5 of approximately 3.8 kb spanning nucleotides 2391 to 6135 of Figure 2;
viii) HRR-7A of approximately 2.2 kb spanning nucleotides 186 to 2396 of Figure 2; and
ix) HRR-7B of approximately 0.4 kb spanning nucleotides 104 to 549 of Figure 2.

7. A selected said DNA probe of claim 6 for use in conducting RFLP analysis of families to detect members of such family who are susceptible to malignant hyperthermia.

8. A cloning vector including a fragment or the entire DNA sequence of claim 1, 2, or 3.

9. A suitable host adapted for expressing DNA and producing a corresponding encoded protein, said host being characterized by a cloning vector including a fragment of or the entire DNA sequence of claim 1, 2, or 3.

10. Human ryanodine receptor protein or amino acid fragments thereof when produced by culturing a suitable host which is adapted to express a recombinant vector which includes a fragment of or the entire DNA sequence of claim 1, 2, or 3.

11. Substantially purified human ryanodine receptor protein which is free of any foreign human protein having a molecular weight of approximately 563,000 daltons and an amino acid sequence of Figure 2.

12. A protein fragment free of foreign human protein having an amino acid sequence which is a portion of said

amino acid sequence of said human ryanodine receptor of claim 11.

13. A substantially purified antibody specific to said protein fragment of claim 12.

14. An antibody of claim 13 which is a polyclonal antibody.

15. An antibody of claim 13 which is a monoclonal antibody.

16. A diagnostic DNA assay for determining susceptibility of a person to malignant hyperthermia where said person is from a family of one or more indivudals who is susceptible to malignant hyperthermia, said assay comprising the steps of:

i) isolating DNA from at least chromosome 19 of a person being tested;

ii) performing RFLP analysis on said isolated chromosome 19 DNA and using selected DNA probes of claim 6 as RFLP markers in said RFLP analysis;

iii) detecting linkage with RFLP analysis of an affected family individual to determine if said person is susceptible to malignant hyperthermia.

17. The use of one or more selected DNA probes of claim 6 for determining malignant hyperthermia in a pig.

18. The use of an antibody of claim 13 in an immunoassay to determine if a subject is susceptible to malignant hyperthermia.

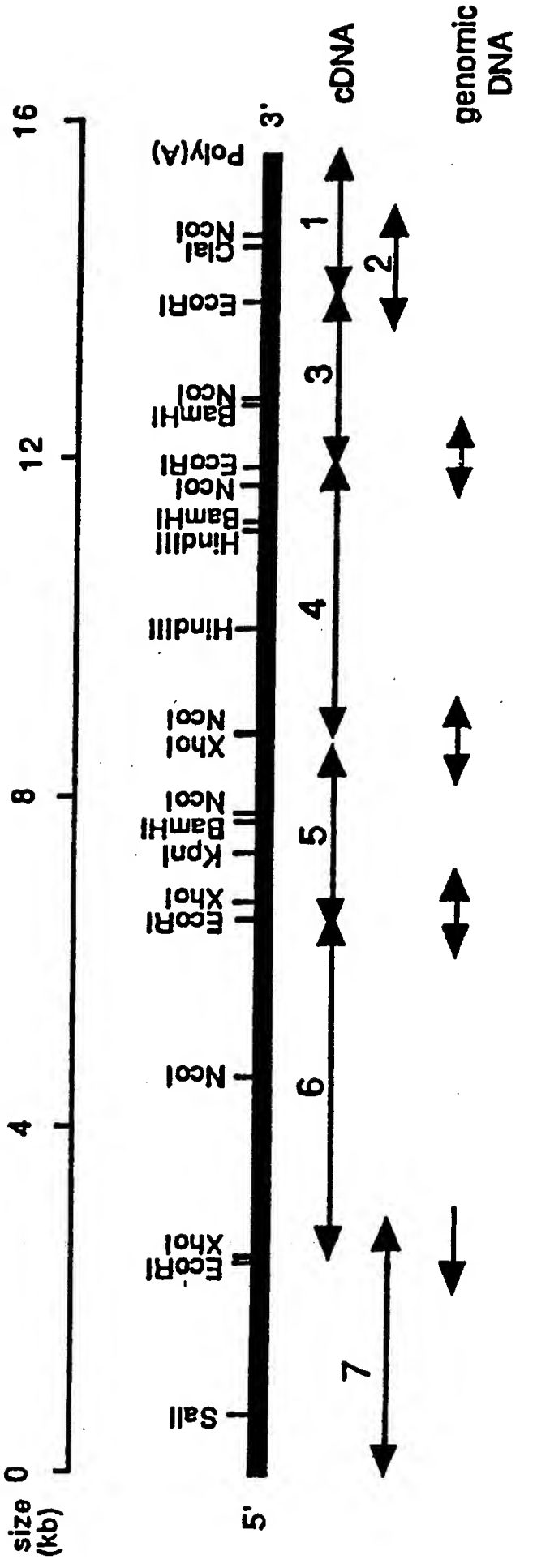
cDNA
genomic DNA
3'
Poly(A)
ClaI
NcoI
EcoRI
BamHI
NcoI
EcoRI
NcoI
BamHI
HindIII
HindIII
NcoI
XhoI
NcoI
BamHI
KpnI
EcoRI
XhoI
NcoI
EcoRI
XhoI
SalI
5'
1
2
3
4
5
6
7
size 0 (kb)
4
8
12
16

FIG.1.

FIG. 2.

GTCTCCGAGGTCTCCGACCCCGCCCCGCGCCCAGCCCTCCCGCCCAGCCCGCAGCCCCCTCCCTCTGTTCCCCGACCTCAGACCCTGGGCTTCCGACCTCGACATC -1

ATGGGTGACGCAGAAGGCGAAGACGAGGTCCAGTTCCTGCGGACGGACGATGAGGTGGTCCTGCAGTGCAGCGCTACCGTGCTCAAGGAGCAGCTCAAGCTCTGCCTGGCCGCCGAGGGC 120
MetGlyAspAlaGluGlyGluAspGluValGlnPheLeuArgThrAspAspGluValValLeuGlnCysSerAlaThrValLeuLysGluGlnLeuLysLeuCysLeuAlaAlaGluGly 40

TTCGGCAACCGCCTGTGCTTCCTGGAGCCCACTAGCAACGCGCAGAATGTGCCCCCCGATCTGGCCATCTGTTGCTTCGTCCTGGAGCAGTCCCTGTCTGTGCGAGCCCTGCAGGAGATG 240
PheGlyAsnArgLeuCysPheLeuGluProThrSerAsnAlaGlnAsnValProProAspLeuAlaIleCysCysPheValLeuGluGlnSerLeuSerValArgAlaLeuGlnGluMet 80

CTGGCTAACACGGTGGAGGCTGGCGTGGAGTCATCCCAGGGCGGGGGACACAGGACGCTCCTGTATGGCCATGCCATCCTGCTCCGGCATGCACACAGCCGCATGTATCTGAGCTGCCTC 360
LeuAlaAsnThrValGluAlaGlyValGluSerSerGlnGlyGlyGlyHisArgThrLeuLeuTyrGlyHisAlaIleLeuLeuArgHisAlaHisSerArgMetTyrLeuSerCysLeu 120

ACCACCTCCCGCTCCATGACTGACAAGCTGGCCTTCGATGTGGGACTGCAGGAGGACGCAACAGGAGAGGCTTGCTGGTGGACCATGCACCCAGCCTCCAAGCAGAGGTCTGAAGGAGAA 480
ThrThrSerArgSerMetThrAspLysLeuAlaPheAspValGlyLeuGlnGluAspAlaThrGlyGluAlaCysTrpTrpThrMetHisProAlaSerLysGlnArgSerGluGlyGlu 160

AAGGTCCGCGTTGGGGATGACATCATCCTTGTCAGTGTCTCCTCCGAGCGCTACCTGCACCTGTCGACCGCCAGTGGGGAGCTCCAGGTTGACGCTTCCTTCATGCAGACACTGTGGAAC 600
LysValArgValGlyAspAspIleIleLeuValSerValSerSerGluArgTyrLeuHisLeuSerThrAlaSerGlyGluLeuGlnValAspAlaSerPheMetGlnThrLeuTrpAsn 200

ATGAACCCCATCTGCTCCCGCTGCGAAGAGGGCTTCGTGACGGGAGGTCACGTCCTCCGCCTCTTTCATGGACATATGGATGAGTGTCTGACCATTTCCCCTGCTGACAGTGATGACCAG 720
MetAsnProIleCysSerArgCysGluGluGlyPheValThrGlyGlyHisValLeuArgLeuPheHisGlyHisMetAspGluCysLeuThrIleSerProAlaAspSerAspAspGln 240

CGCAGACTTGTCTACTATGAGGGGGGAGCTGTGTGCACTCATGCCCGCTCCCTCTGGAGGCTGGAGCCACTGAGAATCAGCTGGAGTGGGAGCCACCTGCGCTGGGGCCAGCCACTCCGA 840
ArgArgLeuValTyrTyrGluGlyGlyAlaValCysThrHisAlaArgSerLeuTrpArgLeuGluProLeuArgIleSerTrpSerGlySerHisLeuArgTrpGlyGlnProLeuArg 280

GTCCGGCATGTCACTACCGGGCAGTACCTAGCGCTCACCGAGGACCAGGGCCTGGTGGTGGTTGACGCCAGCAAGGCTCACACCAAGGCTACCTCCTTCTGCTTCCGCATCTCCAAGGAG 960
ValArgHisValThrThrGlyGlnTyrLeuAlaLeuThrGluAspGlnGlyLeuValValValAspAlaSerLysAlaHisThrLysAlaThrSerPheCysPheArgIleSerLysGlu 320

AAGCTGGATGTGGCCCCCAAGCGGGATGTGGAGGGCATGGGCCCCCCTGAGATCAAGTACGGGGAGTCACTGTGCTTCGTGCAGCATGTGGCCTCAGGACTGTGGCTCACCTATGCCGCT 1080
LysLeuAspValAlaProLysArgAspValGluGlyMetGlyProProGluIleLysTyrGlyGluSerLeuCysPheValGlnHisValAlaSerGlyLeuTrpLeuThrTyrAlaAla 360

CCAGACCCCAAGGCCCTGCGGCTCGGCGTGCTCAAGAAGAAGGCCATGCTGCACCAGGAGGGCCACATGGACGACGCACTGTCGCTGACCCGCTGCCAGCAGGAGGAGTCCCAGGCCGCC 1200
ProAspProLysAlaLeuArgLeuGlyValLeuLysLysLysAlaMetLeuHisGlnGluGlyHisMetAspAspAlaLeuSerLeuThrArgCysGlnGlnGluGluSerGlnAlaAla 400

CGCATGATCCACAGCACCAATGGCCTATACAACCAGTTCATCAAGAGCCTGGACAGCTTCAGCGGGAAGCCACGGGGCTCGGGGCCACCCGCTGGCACGGCGCTGCCCATCGAGGGCGTT 1320
ArgMetIleHisSerThrAsnGlyLeuTyrAsnGlnPheIleLysSerLeuAspSerPheSerGlyLysProArgGlySerGlyProProAlaGlyThrAlaLeuProIleGluGlyVal 440

ATCCTGAGCCTGCAGGACCTCATCATCTACTTCGAGCCTCCCTCCGAGGACTTGCAGCACGAGGAGAAGCAGAGCAAGCTGCGAAGCCTGCGCAACCGCCAGAGCCTCTTCCAGGAGGAG 1440
IleLeuSerLeuGlnAspLeuIleIleTyrPheGluProProSerGluAspLeuGlnHisGluGluLysGlnSerLysLeuArgSerLeuArgAsnArgGlnSerLeuPheGlnGluGlu 480

GGGATGCTCTCCATGGTCCTGAATTGCATAGACCGCCTAAATGTCTACACCACTGCTGCCCACTTTGCTGAGTTTGCAGGGGAGGAGGCAGCCGAGTCCTGGAAAGAGATTGTGAATCTT 1560
GlyMetLeuSerMetValLeuAsnCysIleAspArgLeuAsnValTyrThrThrAlaAlaHisPheAlaGluPheAlaGlyGluGluAlaAlaGluSerTrpLysGluIleValAsnLeu 520

CTCTATGAACTCCTAGCTTCTCTAATCCGTGGCAATCGTAGCAACTGTGCCCTCTTCTCCACAAACTTGGACTGGCTGGTCAGCAAGCTGGATCGGCTGGAGGCCTCATCTGGCATCCTG 1680
LeuTyrGluLeuLeuAlaSerLeuIleArgGlyAsnArgSerAsnCysAlaLeuPheSerThrAsnLeuAspTrpLeuValSerLysLeuAspArgLeuGluAlaSerSerGlyIleLeu 560

FIG. 2A.

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GAGGTCCTGTACTGTGTCCTCATTGAGAGTCCAGAGGTTCTGAACATCATCCAGGAGAATCACATCAAGTCCATCATCTCCCTCCTGGACAAGCATGGGAGGAACCACAAGGTCCTGGAC  1800
GluValLeuTyrCysValLeuIleGluSerProGluValLeuAsnIleIleGlnGluAsnHisIleLysSerIleIleSerLeuLeuAspLysHisGlyArgAsnHisLysValLeuAsp  600

GTGCTATGCTCCCTGTGTGTCTGTAATGGTGTGGCTGTACGCTCCAACCAAGATCTTATTACTGAGAACTTGCTGCCTGGCCGTGAGCTTCTGCTGCAGACAAACCTCATCAACTATGTC  1920
ValLeuCysSerLeuCysValCysAsnGlyValAlaValArgSerAsnGlnAspLeuIleThrGluAsnLeuLeuProGlyArgGluLeuLeuLeuGlnThrAsnLeuIleAsnTyrVal  640

ACCAGCATCCGCCCCCAACATCTTTGTGGGCCGAGCGGAAGGCACCACGCAGTACAGCAAATGGTACTTTGAGGTGATGGTGGACGAGGTGACTCCATTTCTGACAGCTCAGGCCACCCAC  2040
ThrSerIleArgProAsnIlePheValGlyArgAlaGluGlyThrThrGlnTyrSerLysTrpTyrPheGluValMetValAspGluValThrProPheLeuThrAlaGlnAlaThrHis  680

TTGCGGGTGGGCTGGGCCCTCACCGAGGGCTACACCCCCTACCCTGGCGCCGGCGCAGGCCTGGGGCGGCAACGGGGTCGGCCATGACCTCTATTCCTACGGCTTTGATGGACTGCATCTC  2160
LeuArgValGlyTrpAlaLeuThrGluGlyTyrThrProTyrProGlyAlaGlyGluGlyTrpGlyGlyAsnGlyValGlyAspAspLeuTyrSerTyrGlyPheAspGlyLeuHisLeu  720

TGGACAGGACACGTGGCACGCCCAGTGACTTCCCCAGGGCAGCACCTCCTGGCCCCTGAAGACGTGATCAGCTGCTCCCTGGACCTCAGCGTGCCGTCCATCTCCTTCCGCATCAACGGC  2280
TrpThrGlyHisValAlaArgProValThrSerProGlyGlnHisLeuLeuAlaProGluAspValIleSerCysCysLeuAspLeuSerValProSerIleSerPheArgIleAsnGly  760

TGCCCTGTGCAGGGTGTCTTTGAGTCCTTCAACCTGGACGGGCTCTTCTTCCCTGTTGTCAGCTTCTCGGCTGGTGTCAAGGTGCGGTTCCTCCTGGGTGGCCGCCATGGTGAATTCAAG  2400
CysProValGlnGlyValPheGluSerPheAsnLeuAspGlyLeuPhePheProValValSerPheSerAlaGlyValLysValArgPheLeuLeuGlyGlyArgHisGlyGluPheLys  800

TTCCTGCCCCCCACCTGGCTATGCTCCATGCCATGAGGCTGTGCTCCCTCGAGAGCGACTCCATCTTGAACCCATCAAGGAGTATCGACGGGACGGGCCCCCGGGGGGCCTCACCTGGTGGGC  2520
PheLeuProProProGlyTyrAlaProCysHisGluAlaValLeuProArgGluArgLeuHisLeuGluProIleLysGluTyrArgArgGluGlyProArgGlyProHisLeuValGly  840

CCCAGTCGCTGCCTCTCACACACCGACTTCGTGCCCTGCCCTGTGGACACTGTCCAGATTGTCCTGCCGCCCCATCTGGAGCGCATTCGGCAGAAGCTGGCCGAGAACATCCACGAGCTC  2640
ProSerArgCysLeuSerHisThrAspPheValProCysProValAspThrValGlnIleValLeuProProHisLeuGluArgIleArgGluLysLeuAlaGluAsnIleHisGluLeu  880

TGGGCGCTAACCCGCATCGAGCAGGGCTGGACCTACGGCCCCGGTTCGGGATGACAACAAGAGGCTGCACCCGTGTCTTGTGGACTTCCACAGCCTTCCAGAGCCTGAGAGGAACTACAAC  2760
TrpAlaLeuThrArgIleGluGlnGlyTrpThrTyrGlyProValArgAspAspAsnLysArgLeuHisProCysLeuValAspPheHisSerLeuProGluProGluArgAsnTyrAsn  920

CTGCAGATGTCTGGGGAGACGCTCAAGACTCTGCTGGCTCTGGGCTGCCACGTGGGCATGGCGGATGAGAAGGCGGAGGACAACCTGAAGAAGACAAAACTCCCCAAGACGTATATGATG  2880
LeuGlnMetSerGlyGluThrLeuLysThrLeuLeuAlaLeuGlyCysHisValGlyMetAlaAspGluLysAlaGluAspAsnLeuLysLysThrLysLeuProLysThrTyrMetMet  960

AGCAATGGGTACAAGCCGGCTCCGCTGGACCTGAGCCACGTGCGGCTGACGCCGGCGCAGACAACACTGGTGGACCGTCTGGCAGAAAATGGGCACAACGTGTGGGCCCGAGACCGCGTG  3000
SerAsnGlyTyrLysProAlaProLeuAspLeuSerHisValArgLeuThrProAlaGlnThrThrLeuValAspArgLeuAlaGluAsnGlyHisAsnValTrpAlaArgAspArgVal  1000

GGCCAGGGCTGGAGCTACAGCGCAGTGCAGGACATCCCAGCGCGCCGAAACCCTCGGCTGGTGCCCTACCGCCTGCTGGATGAAGCCACCAAGCGCAGCAACCGGGACAGCCTCTGCCAG  3120
GlyGlnGlyTrpSerTyrSerAlaValGlnAspIleProAlaArgArgAsnProArgLeuValProTyrArgLeuLeuAspGluAlaThrLysArgSerAsnArgAspSerLeuCysGln  1040

GCCGTGCGCACCCTCCTGGGCTACGGCTACAACATCGAGCCTCCTGACCAGGAGCCCAGTCAGGTGGAGAACCAGTCTCGTTGTGACCGGGTGCGCATCTTCCGGGCAGAGAAATCCTAT  3240
AlaValArgThrLeuLeuGlyTyrGlyTyrAsnIleGluProProAspGlnGluProSerGlnValGluAsnGlnSerArgCysAspArgValArgIlePheArgAlaGluLysSerTyr  1080

ACAGTCCAGAGCGGCCGCCTCGTACTTCGAGTTTGAACCAGTCACCACAGGCGAGATGCGCGTGGGCTGGGCGAGGCCCGAGCTGAGGCCTGATGTAGACCTGGGAGCCTGACGACCTGGCC  3360
ThrValGlnSerGlyArgTrpTyrPheGluPheGluAlaValThrThrGlyGluMetArgValGlyTrpAlaArgProGluLeuArgProAspValGluLeuGlyAlaAspGluLeuAla  1120
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FIG. 2B.

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TATGTCTTCAATGGGCACCGCGGCCCAGCGCTGGCACTTGGCCAGTGAACCATTTGGGCGCCCCTGGCAGCCGGGCGATGTCGTTGGCTGTATGATCGACCTCACAGAGAACACCATTATC  3480
TyrValPheAsnGlyHisArgGlyGlnArgTrpHisLeuGlySerGluProPheGlyArgProTrpGlnProGlyAspValValGlyCysMetIleAspLeuThrGluAsnThrIleIle  1160

TTCACCCTCAATGGCGAGGTCCTCATGTCTGACTCAGGCTCCGAAACAGCCTTCCGGGAGATTGAGATTGGGGACGGCTTCCTGCCCCGTCTGCAGCTTGGGACCTGGCCAGGTGGGTCAT  3600
PheThrLeuAsnGlyGluValLeuMetSerAspSerGlySerGluThrAlaPheArgGluIleGluIleGlyAspGlyPheLeuProValCysSerLeuGlyProGlyGlnValGlyHis  1200

CTGAACCTGGGCCAGGACGTGAGCTCTCTGAGGTTCTTTGCCATCTGTGGCCTCCAGGAAGGCTTCGAGCCATTTGCCATCAACATGCAGCGCCCAGTCACCACCTGGTTCAGCAAAGCC  3720
LeuAsnLeuGlyGlnAspValSerSerLeuArgPhePheAlaIleCysGlyLeuGlnGluGlyPheGluProPheAlaIleAsnMetGlnArgProValThrThrTrpPheSerLysGly  1240

CTGCCCCAGTTTGAGCCAGTCCCCCTTGAACACCCTCACTATGAGGTATCCCGAGTGGACGGCACTGTGGACACGCCCCCCTGCCTGCGCCTGACCCACCGCACCTGGGGCTCCCAGAAC  3840
LeuProGlnPheGluProValProLeuGluHisProHisTyrGluValSerArgValAspGlyThrValAspThrProProCysLeuArgLeuThrHisArgThrTrpGlySerGlnAsn  1280

ACCCTGGTGCAGATGCTTTTCCTGCGGCTGAGCCTCCCAGTCCAGTTCCACCAGCACTTCCGCTGCACTGCAGGGCCCACCCCGCTGGCACCTCCTGGCCTGCAGCCCCCCGCCGAGGAC  3960
SerLeuValGluMetLeuPheLeuArgLeuSerLeuProValGlnPheHisGlnHisPheArgCysThrAlaGlyAlaThrProLeuAlaProProGlyLeuGlnProProAlaGluAsp  1320

GAGGCCCCGGCGGCCGGAACCCGACCCTGACTACGAAAACCTGCGCCGCTCAGCTGGGGGCTGGAGCGAGGCAGAGAACGGCAAAGAAGGGACTGCGAAGGAGGGCGCCCCCGGGGGCACC  4080
GluAlaArgAlaAlaGluProAspProAspTyrGluAsnLeuArgArgSerAlaGlyGlyTrpSerGluAlaGluAsnGlyLysGluGlyThrAlaLysGluGlyAlaProGlyGlyThr  1360

CCGCAGGCCGCCGAGAGGCCCACCGGCCAGGGCGGAGAATGAGAAGGATGCCACCACCGAGAAGAACAAGAAGAGAGGCTTCTTATTCAAGGCCAAGAAGGTCGCCATGATGACCCAGCCA  4200
ProGlnAlaGlyArgGlyAlaProAlaArgAlaGluAsnGluLysAspAlaThrThrGluLysAsnLysLysArgGlyPheLeuPheLysAlaLysLysValAlaMetMetThrGlnPro  1400

CCGGCCACCCCCACGCTGCCCCGACTCCCTCACGACGTGGTGCCTGCAGACAACCGCCATGACCCCGAGATCATCCTCAACACCACCACGTACTATTACTCCGTGAGGGTCTTTGCTGGA  4320
ProAlaThrProThrLeuProArgLeuProHisAspValValProAlaAspAsnArgAspAspProGluIleIleLeuAsnThrThrThrTyrTyrTyrSerValArgValPheAlaGly  1440

CAGGAGCCCAGCTGCGTGTGGGCGGGCTGGGTCACCCCTGACTACCATCAGCACGACATGAGCTTCGACCTCAGCAAGGTCCGGGTCGTGACGGTGACCATGGGGGATGAACAAGGCAAC  4440
GlnGluProSerCysValTrpAlaGlyTrpValThrProAspTyrHisGlnHisAspMetSerPheAspLeuSerLysValArgValValThrValThrMetGlyAspGluGlnGlyAsn  1480

GTCCACAGCAGCCTCAAGTGTAGCAACTGCTACATGGTGTGGGGCGGAGACTTTGTGAGTCCCGGGCAGCAGGGCCGGATCAGCCACACGGACCTTGTCATTGGGTGCCTGGTGGACTTG  4560
ValHisSerSerLeuLysCysSerAsnCysTyrMetValTrpGlyGlyAspPheValSerProGlyGlnGlnGlyArgIleSerHisThrAspLeuValIleGlyCysLeuValAspLeu  1520

GCCACTGGCTTAATGACCTTTACAGCCAATGGCAAAGAGAGCAACACCTTTTTCCAGGTGGAACCCAACACTAAGCTATTTCCTGCCGTCTTCGTCCTGCCCACCCACCAGAACGTCATC  4680
AlaThrGlyLeuMetThrPheThrAlaAsnGlyLysGluSerAsnThrPhePheGlnValGluProAsnThrLysLeuPheProAlaValPheValLeuProThrHisGlnAsnValIle  1560

CAGTTTGAGCTGGGGAAGCAGAAGAACATCATGCCGTTGTCAGCCGCCATGTTCCAAAGCGAGCGCAAGAACCCGGCCCCGCAGTGCCCACCGCGGCTGGAGATGCAGATGCTGATGCCA  4800
GlnPheGluLeuGlyLysGlnLysAsnIleMetProLeuSerAlaAlaMetPheGlnSerGluArgLysAsnProAlaProGlnCysProProArgLeuGluMetGlnMetLeuMetPro  1600

GTGTCCTGGAGCCCCATGCCCAACCACTTCCTGCAGGTGGAGACGAGGCGTGCCGGCGAGCGGCTGGGCTGGGCCGTGCAGTGCCAGGAGCCGCTCACCATGATGGCGCTGCACATCCCC  4920
ValSerTrpSerArgMetProAsnHisPheLeuGlnValGluThrArgArgAlaGlyGluArgLeuGlyTrpAlaValGlnCysGlnGluProLeuThrMetMetAlaLeuHisIlePro  1640

GAGGAGAACCGGTGCATGGACATCCTGGAGCTGTCGGAGCGCCTGGACCTGCAGCGCTTCCACTCGCACACCCTGCGCCTCTACCGCGCTGTGTGCGCCCTGGGCAACAATCGCGTGGCG  5040
GluGluAsnArgCysMetAspIleLeuGluLeuSerGluArgLeuAspLeuGlnArgPheHisSerHisThrLeuArgLeuTyrArgAlaValCysAlaLeuGlyAsnAsnArgValAla  1680
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FIG. 2C.

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CACGCTCTGTGCAGCCACGTAGACCAAGCTCAGCTGCTGCACGCCCTGGAGGACGCGCACCTGCCAGGCCCACTGCGCGCAGGCTACTATGACCTCCTCATCAGCATCCACCTCGAAAGT  5160
HisAlaLeuCysSerHisValAspGlnAlaGlnLeuLeuHisAlaLeuGluAspAlaHisLeuProGlyProLeuArgAlaGlyTyrTyrAspLeuLeuIleSerIleHisLeuGluSer  1720

GCCTGCCGCAGCCGCCGCTCCATGCTCTCTGAATACATCGTGCCCCTCACGCCTGAGACCCGCGCCATCACGCTCTTCCCTCCTGGAAGGAGCACAGAAAATGGTCACCCCCGGCATGGC  5280
AlaCysArgSerArgArgSerMetLeuSerGluTyrIleValProLeuThrProGluThrArgAlaIleThrLeuPheProProGlyArgSerThrGluAsnGlyHisProArgHisGly  1760

CTGCCGGGAGTTGGAGTCACCACTTCGCTGAGGCCCCCCGCATCATTTCTCGCCCCCCTGTTTCGTGGCCGCTCTGCCAGCTGCTGGGGCAGCAGAGGCCCCGGCCCGCCTCAGCCCTGCC  5400
LeuProGlyValGlyValThrThrSerLeuArgProProHisHisPheSerProProCysPheValAlaAlaLeuProAlaAlaGlyAlaAlaGluAlaProAlaArgLeuSerProAla  1800

ATCCCGCTGGAGGCCCTGCGGGACAAGGCACTGAGGATGCTGGGGGAGGCGGTCGCGCGACGGTGGGCAGCACGCTCGCGACCCCGTCGGGGCCTCCGTGGAGTTCCAGTTTGTGCCCTGTG  5520
IleProLeuGluAlaLeuArgAspLysAlaLeuArgMetLeuGlyGluAlaValArgAspGlyGlyGlnHisAlaArgAspProValGlyAlaSerValGluPheGlnPheValProVal  1840

CTCAAGCTCGTGTCCACCCTGCTGGTGATGGGCATCTTTGGCGATGAGGATGTGAAACAGATCTTGAAGATGATTGAGCCTGAGGTCTTCACTGAGGAAGAAGAGGAGGAGGACGAGGAG  5640
LeuLysLeuValSerThrLeuLeuValMetGlyIlePheGlyAspGluAspValLysGlnIleLeuLysMetIleGluProGluValPheThrGluGluGluGluGluGluAspGluGlu  1880

GAAGAGGGTGAAGAGGAAGATGAGGAGGAGAAGGAGGAGGATGAGGAGGAAACAGCACAGGAAAAGGAAGATGAGGAAAAAGAGGAAGAGGAGGCAGCAGAAGGGGAGAAAGAAGAAGGC  5760
GluGluGlyGluGluGluAspGluGluGluLysGluGluAspGluGluGluThrAlaGlnGluLysGluAspGluGluLysGluGluGluGluAlaAlaGluGlyGluLysGluGluGly  1920

TTGGAGGAAGGGCTGCTCCAGATGAAGTTGCCAGAGTCTGTGAAGTTACAGATGTGCCCACCTGCTGGAGTATTTCTGTGACCAAGAGCTGCAGCACCGTGTGGAGTCCCTGGCAGCCTTT  5880
LeuGluGluGlyLeuLeuGlnMetLysLeuProGluSerValLysLeuGlnMetCysHisLeuLeuGluTyrPheCysAspGlnGluLeuGlnHisArgValGluSerLeuAlaAlaPhe  1960

GCGGAGCGCTATGTGGACAAGCTCCAGGCCAACCAGCGGAGCCGCTATGGCCTCCTCATAAAAGCCTTCAGCATGACCGCAGCAGAGACTGCAAGACGTACCCGCGAGTTCCGCTCCCCA  6000
AlaGluArgTyrValAspLysLeuGlnAlaAsnGlnArgSerArgTyrGlyLeuLeuIleLysAlaPheSerMetThrAlaAlaGluThrAlaArgArgThrArgGluPheArgSerPro  2000

CCCCAGGAACAGATCAATATGCTATTGCAATTCAAAGATGGTACAGATGAGGAAGACTGTCCTCTCCCTGAAGAGATTCGACAGGATTTGCTTGACTTTCATCAAGACCTGCTGGCACAC  6120
ProGlnGluGlnIleAsnMetLeuLeuGlnPheLysAspGlyThrAspGluGluAspCysProLeuProGluGluIleArgGlnAspLeuLeuAspPheHisGlnAspLeuLeuAlaHis  2040

TGTGGAATTCAGCTAGATGGAGAGGAGGAGGAACCAGAGGAAGAGACCACCCTGGGCAGCCGCCTCATGAGCCTGTTGGAGAAAGTGCGGCTGGTGAAGAAGAAGGAAGAGAAACCTGAG  6240
CysGlyIleGlnLeuAspGlyGluGluGluGluProGluGluGluThrThrLeuGlySerArgLeuMetSerLeuLeuGluLysValArgLeuValLysLysLysGluGluLysProGlu  2080

GAGGAGCCGGTCAGCAGAGGAGAGCAAACCCCCGTCCCTGCAGGAGCTGGTGTCCCACATGGTGGTGCGCTGGGCCCAAGAGGACTTCGTGCAGAGCCCCGAGCTGGTGCGGGCCATGTTC  6360
GluGluArgSerAlaGluGluSerLysProArgSerLeuGlnGluLeuValSerHisMetValValArgTrpAlaGlnGluAspPheValGlnSerProGluLeuValArgAlaMetPhe  2120

AGCCTCCTGCACCGGCAGTACGACGGGCTGGGTGAGCTGCTGCGTGCCCTGCCGCGGGCGTACACCATCTCACCGTCCTCCGTGGAAGACACCATGAGCCTGCTCGAGTGCCTCGGCCAG  6480
SerLeuLeuHisArgGlnTyrAspGlyLeuGlyGluLeuLeuArgAlaLeuProArgAlaTyrThrIleSerProSerSerValGluAspThrMetSerLeuLeuGluCysLeuGlyGln  2160

ATCCGCTCGCTGCTCATCGTGCAGATGGGCCCCCAGGAGGAGAACCTCATGATCCAGAGCATCGGGAACATCATGAACAACAAAGTCTTCTACCAACACCCGAACCTGATGACGGCGCTG  6600
IleArgSerLeuLeuIleValGlnMetGlyProGlnGluGluAsnLeuMetIleGlnSerIleGlyAsnIleMetAsnAsnLysValPheTyrGlnHisProAsnLeuMetArgAlaLeu  2200

GGCATGCACGAGACGGTCATGGAGGTCATGGTCAACGTCCTCGGGGGCCGGCGAGTCCAAGGAGATCCGCTTCCCCAAGATGGTGACAAGCTGCTGCCGCTTCCTCTGCTATTTCTGCCGA  6720
GlyMetHisGluThrValMetGluValMetValAsnValLeuGlyGlyGlyGluSerLysGluIleArgPheProLysMetValThrSerCysCysArgPheLeuCysTyrPheCysArg  2240
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FIG. 2D.

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ATCAGCCGGCAGAACCAGCGCTCCATGTTTGACCACCTGAGCTACCTGCTGGAGAACAGTGGCATCGGCCTGGGCATGCAGGGCTCCACGCCCCCTGGACGTGGCTGCTGCCTCCGTCATT  6840
IleSerArgGlnAsnGlnArgSerMetPheAspHisLeuSerTyrLeuLeuGluAsnSerGlyIleGlyLeuGlyMetGlnGlySerThrProLeuAspValAlaAlaAlaSerValIle  2280

GACAACAATGAGCTGGCCTTGGCATTGCAGGAGCAGGACCTGGAAAAGGTTGTGTCCTACCTGGCAGGCTGTGGCCTCCAGAGCTGCCCCATGCTTGTGGCCAAAGGGTACCCAGACATT  6960
AspAsnAsnGluLeuAlaLeuAlaLeuGlnGluGlnAspLeuGluLysValValSerTyrLeuAlaGlyCysGlyLeuGlnSerCysProMetLeuValAlaLysGlyTyrProAspIle  2320

GGCTGGAAGCCCTGTGGTGGAGAGCGCTACCTGGACTTCCTGCGCTTTGCTGTCTTCGTCAACGGCGAGACGTGGAGGAGAACGCCAATGTGGTGGTGCGGCTGCTCATCCGGAAGCCT  7080
GlyTrpLysProCysGlyGlyGluArgTyrLeuAspPheLeuArgPheAlaValPheValAsnGlyGluSerValGluGluAsnAlaAsnValValValArgLeuLeuIleArgLysPro  2360

GAGTGCTTCGGACCCGCCCTGCGGGGTGAGGGTGGCTCAGGGCTGCTGGCTGCCATCGAAGAGGCCATCCGCATCTCCGAGGACCCTGCGAGGGATGGCCCAGGCATCCGCAGGGACCGG  7200
GluCysPheGlyProAlaLeuArgGlyGluGlySerGlyLeuLeuAlaAlaIleGluGluAlaIleArgIleSerGluAspProAlaArgAspGlyProGlyIleArgArgAspArg  2400

CGGCGCGAGCACTTTGGTGAGGAACCGCCTGAAGAAAACCGGGTGCACCTGGGACACGCCATCATGTCCTTCTATGCCGCCTTGATCGACCTGCTCGGACGCTGTGCACCAGAGATGCAT  7320
ArgArgGluHisPheGlyGluGluProProGluGluAsnArgValHisLeuGlyHisAlaIleMetSerPheTyrAlaAlaLeuIleAspLeuLeuGlyArgCysAlaProGluMetHis  2440

CTAATCCAAGCCGGCAAGGGTGAGGCCCTGCGGATCCGCGCCATCCTCCGCTCCCTTGTGCCCTTGGAGGACCTTGTGGCCATCATCACCCTCCCACTGCAGATTCCCACCCTGGGCAAA  7440
LeuIleGlnAlaGlyLysGlyGluAlaLeuArgIleArgAlaIleLeuArgSerLeuValProLeuGluAspLeuValGlyIleIleSerLeuProLeuGlnIleProThrLeuGlyLys  2480

GATGGGGCTCTGGTGCAGCCAAAGATGTCAGCATCCTTCGTGCCGGACCACAAGGCGTCCATGGTGCTCTTCCTGGACCGTGTGTATGGCATCGAGAACCAGGACTTCTTGCTGCACGTG  7560
AspGlyAlaLeuValGlnProLysMetSerAlaSerPheValProAspHisLysAlaSerMetValLeuPheLeuAspArgValTyrGlyIleGluAsnGlnAspPheLeuLeuHisVal  2520

CTGGACGTGGGGTTCCTGCCCCGACATGAGGGCAGCCGCCTCCCTGGACACGGCCACTTTCAGCACCACCGAGATGGCGCTGGCCGTGAACCGCTACCTGTGCCTGGCCGTGCTGCCGCTC  7680
LeuAspValGlyPheLeuProAspMetArgAlaAlaAlaSerLeuAspThrAlaThrPheSerThrThrGluMetAlaLeuAlaValAsnArgTyrLeuCysLeuAlaValLeuProLeu  2560

ATCACCAAGTGTGCGCCCGCTCTTTGCGGGCACAGAACACCGCGCCATCATGGTGGACTCTATGCTGCATACCGTGTACCGCCTGTCTCGGGGTCGTTCGCTCACCAAGGCGCAGCGTGAC  7800
IleThrLysCysAlaProLeuPheAlaGlyThrGluHisArgAlaIleMetValAspSerMetLeuHisThrValTyrArgLeuSerArgGlyArgSerLeuThrLysAlaGlnArgAsp  2600

GTCATCGAGGACTGCCTCATGTCGCTCTGCAGGTACATCCGCCCCGTCGATGCTGCAGCACCTGTTGCGCCGCCTGGTGTTCGACGTCCCCATCCTCAACGAGTTCGCCAAGATGCCACTC  7920
ValIleGluAspCysLeuMetSerLeuCysArgTyrIleArgProSerMetLeuGlnHisLeuLeuArgArgLeuValPheAspValProIleLeuAsnGluPheAlaLysMetProLeu  2640

AAGCTCCTCACCAACCACTATGAGCCCTGTTGGAAGTACTACTGCCTACCCACGGGCTGGGCCAACTTCGGGGTCACCTCAGAGGAGGAGCTGCACCTCACACGGAAACTCTTCTGGGGC  8040
LysLeuLeuThrAsnHisTyrGluArgCysTrpLysTyrTyrCysLeuProThrGlyTrpAlaAsnPheGlyValThrSerGluGluGluLeuHisLeuThrArgLysLeuPheTrpGly  2680

ATCTTTGACTCTCTGGCCCATAAGAAATACGACCCGGAGCTGTACCGCATGGCCATGCCTTGTCTGTGCGCCATTGCCGGGGCTCTGCCCCCCCGACTATGTGGATGCCTCATACTCATCT  8160
IlePheAspSerLeuAlaHisLysLysTyrAspProGluLeuTyrArgMetAlaMetProCysLeuCysAlaIleAlaGlyAlaLeuProProAspTyrValAspAlaSerTyrSerSer  2720

AAGGCAGAGAAAAAGCCCCACAGTGGATGCTGAAGGCAACTTTGATCCCCGGCCTGTGGAGACCCTCAATGTGATCATCCCGGAGAAGCTGGACTCCTTCATTAACAAGTTTGCGGAGTAC  8280
LysAlaGluLysLysAlaThrValAspAlaGluGlyAsnPheAspProArgProValGluThrLeuAsnValIleIleProGluLysLeuAspSerPheIleAsnLysPheAlaGluTyr  2760

ACACACGAGAAGTGGGCCTTCGACAAGATCCAGAACAACTGGTCCTATGGAGAGAACATAGACGAGGAGCTGAAGACCCACCCCATGCTGAGGCCCTACAAGACCTTTTCAGAGAAGGAC  8400
ThrHisGluLysTrpAlaPheAspLysIleGlnAsnAsnTrpSerTyrGlyGluAsnIleAspGluGluLeuLysThrHisProMetLeuArgProTyrLysThrPheSerGluLysAsp  2800
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FIG. 2E.

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AAAGAGATTTACCGCTGGCCCATCAAGGAGTCCCTGAAGCCCATGATTGCCTGGGAATGGACGATAGAGAAGGCCAGGGAGGGTGAGGAGGAGAAGACGGAAAAGAAAAAAACGGCGAAG  8520
LysGluIleTyrArgTrpProIleLysGluSerLeuLysAlaMetIleAlaTrpGluTrpThrIleGluLysAlaArgGluGlyGluGluGluLysThrGluLysLysLysThrAlaLys  2840

ATATCACAAAGTGCCCAGACCTATGATCCTCGAGAAGGCTACAACCCTCAGCCCCCCCGACCTTAGTGCTGTTACCCTGTCCCGGGAGCTGCAGGCCATGGCAGAACAACTGGCAGAAAAT  8640
IleSerGlnSerAlaGlnThrTyrAspProArgGluGlyTyrAsnProGlnProProAspLeuSerAlaValThrLeuSerArgGluLeuGlnAlaMetAlaGluGlnLeuAlaGluAsn  2880

TACCACAACACGTGGGGACGGAAGAAGAAGCAGGAGCTGGAAGCCAAAGGCGGTGGGACCCACCCCCTGCTGGTCCCCTACGACACGCTCACGGCCAAGGAGAAGGCACGAGATCGAGAG  8760
TyrHisAsnThrTrpGlyArgLysLysLysGlnGluLeuGluAlaLysGlyGlyGlyThrHisProLeuLeuValProTyrAspThrLeuThrAlaLysGluLysAlaArgAspArgGlu  2920

AAGGCCCAGGAGCTACTGAAATTCCTGCAGATGAATGGCTACGCGGTTACAAGAGGCCTTAAGGACATGGAACTGGACTCGTCTTCCATTGAAAAGCGGTTTGCCTTTGGCTTCCTGCAG  8880
LysAlaGlnGluLeuLeuLysPheLeuGlnMetAsnGlyTyrAlaValThrArgGlyLeuLysAspMetGluLeuAspSerSerSerIleGluLysArgPheAlaPheGlyPheLeuGln  2960

CAGCTGCTGCGCTGGATGGACATTTCTCAGGAGTTCATTGCCCACCTGGAGGCTGTGGTCAGCAGTGGGCGAGTGGAAAAGTCCCCACATGAACAGGAGATTAAATTCTTTGCCAAGATC  9000
GlnLeuLeuArgTrpMetAspIleSerGlnGluPheIleAlaHisLeuGluAlaValValSerSerGlyArgValGluLysSerProHisGluGlnGluIleLysPhePheAlaLysIle  3000

CTGCTCCCTTTGATCAACCAGTACTTCACCAACCACTGCCTCTATTTCTTGTCCACTCCGGCTAAAGTGCTGGGCAGCGGTGGCCACGCCTCTAACAAGGAGAAGGAAATGATCACCAGC  9120
LeuLeuProLeuIleAsnGlnTyrPheThrAsnHisCysLeuTyrPheLeuSerThrProAlaLysValLeuGlySerGlyGlyHisAlaSerAsnLysGluLysGluMetIleThrSer  3040

CTCTTCTGCAAACTTGCTGCTCTCGTCCGCCACCGAGTCTCTCTCTTTGGGACAGACGCCCCAGCTGTGGTCAACTGTCTTCACATCCTGGCCCGCTCCCTGGATGCCAGGACAGTGATG  9240
LeuPheCysLysLeuAlaAlaLeuValArgHisArgValSerLeuPheGlyThrAspAlaProAlaValValAsnCysLeuHisIleLeuAlaArgSerLeuAspAlaArgThrValMet  3080

AAGTCAGGCCCTGAGATCGTGAAGGCTGGCCTCCGCTCCTTCTTCGAGAGTGCCTCGGAGGACATCGAGAAGATGGTGGAGAACCTGCGGCTGGGCAAGGTGTCGCAGGCGCCCACCCAG  9360
LysSerGlyProGluIleValLysAlaGlyLeuArgSerPhePheGluSerAlaSerGluAspIleGluLysMetValGluAsnLeuArgLeuGlyLysValSerGlnAlaArgThrGln  3120

GTGAAAGGCGTGGGCCAGAACCTCACCTACACCACTGTGGCACTGCTGCCGGTCCTCACCACCCTCTTCCAGCACATCGCCCAGCACCAGTTCGGAGATGACGTCATCCTGGACGACGTC  9480
ValLysGlyValGlyGlnAsnLeuThrTyrThrThrValAlaLeuLeuProValLeuThrThrLeuPheGlnHisIleAlaGlnHisGlnPheGlyAspAspValIleLeuAspAspVal  3160

CAGGTCTCTTGCTACCGAACCCTGTGCAGTATCTACTCCCTGCGAACCACCAAGAACACTTATGTGGAAAAGCTTCGGCCAGCCCTCGGGGAGTGCCTGGCCCGTCTGGCAGCAGCCATG  9600
GlnValSerCysTyrArgThrLeuCysSerIleTyrSerLeuGlyThrThrLysAsnThrTyrValGluLysLeuArgProAlaLeuGlyGluCysLeuAlaArgLeuAlaAlaAlaMet  3200

CCGGTGGCGTTCCTGGAGCCCGCAGCTGAACGAGTACAACGCCTGCTCCGTGTACACCACCAAGTCTCCGCGGGACCGGCCCATCCTGGCGCTCCCCAACAGTGTGGAGGAGATGTGTCCC  9720
ProValAlaPheLeuGluProGlnLeuAsnGluTyrAsnAlaCysSerValTyrThrThrLysSerProArgGluArgAlaIleLeuGlyLeuProAsnSerValGluGluMetCysPro  3240

GACATCCCCGTGCTGGAGCGGCTCATGGCAGACATTGGGGGGCTGGCCGAGTCAGGTGCCCGCTACACAGAGATGCCGCATGTCATCGAGATCACGCTGCCCATGCTATGCAGCTACCTG  9840
AspIleProValLeuGluArgLeuMetAlaAspIleGlyGlyLeuAlaGluSerGlyAlaArgTyrThrGluMetProHisValIleGluIleThrLeuProMetLeuCysSerTyrLeu  3280

CCCCGATGGTGGGAGCGCGGGGCCCGAGGCACCCCCTTCCGCCCTGCCCGCCGGCGCCCCCCCACCCTGCACAGCTGTCACCTCTGACCACCTCAACTCCCTGCTGGGGAATATCCTGAGA  9960
ProArgTrpTrpGluArgGlyProGluAlaProProSerAlaLeuProAlaGlyAlaProProProCysThrAlaValThrSerAspHisLeuAsnSerLeuLeuGlyAsnIleLeuArg  3320

ATCATCGTCAACAACCTGGGCATTGACGAGGCCTCCTGGATGAAGCGGCTGGCTGTGTTCGCACAGCCCATTGTGAGCCGTGCACGGCCGGAGCTCCTGCAGTCCCACTTCATCCCAACT  10080
IleIleValAsnAsnLeuGlyIleAspGluAlaSerTrpMetLysArgLeuAlaValPheAlaGlnProIleValSerArgAlaArgProGluLeuLeuGlnSerHisPheIleProThr  3360

ATCGGGCGGCTGCGCAAGAGGGCAGGGAAGGTGGTGTCCGAGGAGGAGCAGCTGGCCCTGGAGGCCAAGGCGGAGGCCCAGGAGGGCGAGCTGCTGGTGCGGGACGAGTTCTCTGTGCTC  10200
IleGlyArgLeuArgLysArgAlaGlyLysValValSerGluGluGlnLeuAlaLeuGluAlaLysAlaGluAlaGlnGluGlyGluLeuLeuValArgAspGluPheSerValLeu  3400
```

FIG. 2F.

```
TGCCGGGACCTCTACGCCCTGTATCCGCTGCTCATCCGCTACGTGGACAACAACAGGGCGCAGTGGCTGACGGAGCCGAATCCCAGCGCGGAGGAGCTGTTCAGGATGGTGGGCGAGATC  10320
CysArgAspLeuTyrAlaLeuTyrProLeuLeuIleArgTyrValAspAsnAsnArgAlaGlnTrpLeuThrGluProAsnProSerAlaGluGluLeuPheArgMetValGlyGluIle  3440

TTCATCTACTGGTCCAAGTCCCACAACTTCAAGCGCGAGCAGCAGAACTTTGTGGTCCAGAATGAGATCAACAACATGTCCTTCCTGACTGCTGACAACAAAAGCAAAATGGCTAAGTCC  10440
PheIleTyrTrpSerLysSerHisAsnPheLysArgGluGluGlnAsnPheValValGlnAsnGluIleAsnAsnMetSerPheLeuThrAlaAspAsnLysSerLysMetAlaLysSer  3480

GGTGGCTCGGACCAGGAACGCACCAAGAAGAAGCGCCGGGGGGGACCGGTACTCTGTGCAGACGTCACTGATCGTGGCCACACTGAAGAAGATGCTGCCCATCGGCCTGAATATGTGTGCG  10560
GlyGlySerAspGlnGluArgThrLysLysLysArgArgGlyAspArgTyrSerValGlnThrSerLeuIleValAlaThrLeuLysLysMetLeuProIleGlyLeuAsnMetCysAla  3520

CCCACCGACCAAGACCTCATCACGCTGGCCAAGACCCGTTACGCCCTGAAAGACACAGATGAGGAGGTCCCGGAATTTCTGCACAACAACCTTCACCTTCAGGGAAAGGTCGAAGGCTCC  10680
ProThrAspGlnAspLeuIleThrLeuAlaLysThrArgTyrAlaLeuLysAspThrAspGluGluValArgGluPheLeuHisAsnAsnLeuHisLeuGlnGlyLysValGluGlySer  3560

CCGTCTCTGCGCTGGCAGATGGCTCTGTACCGGGGCGTCCCGGGTCGCGAGGAGGACGCCGATGACCCCGAGAAAATCGTGCGCAGAGTCCAGGAAGTGTCAGCCGTGCTCTACTACCTG  10800
ProSerLeuArgTrpGlnMetAlaLeuTyrArgGlyValProGlyArgGluGluAspAlaAspAspProGluLysIleValArgArgValGlnGluValSerAlaValLeuTyrTyrLeu  3600

GACCAGACCGAGCACCCTTACAAGTCTAAGAAGGCCCTGTGGCACAAGCTTTTGTCCAAACAGCGCCCGCCGCGGCAGTCGTGGCCTGTTTCCGTATGACGCCCCTGTACAACCTGCCCACG  10920
AspGlnThrGluHisProTyrLysSerLysLysAlaValTrpHisLysLeuLeuSerLysGlnArgArgArgAlaValValAlaCysPheArgMetThrProLeuTyrAsnLeuProThr  3640

CACCGGGCATGTAACATGTTCCTGGAGAGCTACAAGGCTGCATGGATCCTGACTGAAGACCACAGTTTTGAGGACCGCATGATAGATGACCTTTCAAAAGCTGGGGAGCAGGAGGAGGAG  11040
HisArgAlaCysAsnMetPheLeuGluSerTyrLysAlaAlaTrpIleLeuThrGluAspHisSerPheGluAspArgMetIleAspAspLeuSerLysAlaGlyGluGlnGluGluGlu  3680

GAGGAAGAGGTGGAAGAGAAGAAGCCAGACCCCCTGCACCAGTTGGTCCTGCACTTCAGCCGCACTGCCCTGACGGAAAAGAGCAAACTGGATGAGGATTACCTGTACATGGCCTATGCT  11160
GluGluGluValGluGluLysLysProAspProLeuHisGlnLeuValLeuHisPheSerArgThrAlaLeuThrGluLysSerLysLeuAspGluAspTyrLeuTyrMetAlaTyrAla  3720

GATATCATGGCAAAGAGCTGCCACCTGGAGGAGGGAGGGGAGAACGGTGAAGCTGAAGAGGAGGTTGAGGTCTCCTTTGAGGAGAAACAGATGGAGAAGCAGAGGCTCTTGTACCAGCAA  11280
AspIleMetAlaLysSerCysHisLeuGluGluGlyGlyGluAsnGlyGluAlaGluGluGluValGluValSerPheGluGluLysGlnMetGluLysGlnArgLeuLeuTyrGlnGln  3760

GCACGGCTGCACACCCGGGGGGCGGCCGAGATGGTGCTGCAGATGATCAGTGCCTGCAAAGGAGAGACAGGTGCCATGGTGTCCTCCACCCTGAAGCTGGGCATCTCCATCCTCAATGGA  11400
AlaArgLeuHisThrArgGlyAlaAlaGluMetValLeuGlnMetIleSerAlaCysLysGlyGluThrGlyAlaMetValSerSerThrLeuLysLeuGlyIleSerIleLeuAsnGly  3800

GGCAATGCTGAGGTCCAGCAGAAAATGCTGGATTATCTTAAGGACAAGAAGGAAGTTGGCTTCTTCCAGAGTATCCAGGCACTGATGCAAACATGCAGCGTCCTGGATCTCAATGCCTTT  11520
GlyAsnAlaGluValGlnGlnLysMetLeuAspTyrLeuLysAspLysLysGluValGlyPhePheGlnSerIleGlnAlaLeuMetGlnThrCysSerValLeuAspLeuAsnAlaPhe  3840

GAGAGACAGCAACAAGCCCGAGGGGCTGGGCATGGTGAATGAGGATGGCACTGTCATCAATCGCCAGAACGGAGAGAAGGTCATGCCGGATGATGAATTCACACAAGACCTGTTCCGATTC  11640
GluArgGlnAsnLysAlaGluGlyLeuGlyMetValAsnGluAspGlyThrValIleAsnArgGlnAsnGlyGluLysValMetAlaAspAspGluPheThrGlnAspLeuPheArgPhe  3880

CTACAATTGCTCTGTGAGGGGCACAATAATGATTTCCAGAACTACCTACGGACACAGACAGGGAACACGACCACTATTAACATCATCATTTGCACTGTGGACTACCTCCTGCGGCTGCAG  11760
LeuGlnLeuLeuCysGluGlyHisAsnAsnAspPheGlnAsnTyrLeuArgThrGlnThrGlyAsnThrThrThrIleAsnIleIleIleCysThrValAspTyrLeuLeuArgLeuGln  3920

GAATCCATCAGCGACTTCTACTGGTACTACTCCGGGCAAGGATGTCATTGAAGAGCAGGGCAAGAGGAACTTCTCCAAAGCCATGTCGGTGGCTAAGCAGGTGTTCAACAGCCTCACTGAG  11880
GluSerIleSerAspPheTyrTrpTyrTyrSerGlyLysAspValIleGluGluGlnGlyLysArgAsnPheSerLysAlaMetSerValAlaLysGlnValPheAsnSerLeuThrGlu  3960
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FIG. 2G.

```
TACATCCAGGGTCCCTGCACCGGGAACCAGCAGAGCCTGGCGCACAGTCGCCTATGGGACGCAGTGGTGGGATTCCTGCACGTGTTCGCCCCACATGATGATGAAGCTCGCTCAGGACTCA 12000
TyrIleGlnGlyProCysThrGlyAsnGlnGlnSerLeuAlaHisSerArgLeuTrpAspAlaValValGlyPheLeuHisValPheAlaHisMetMetMetLysLeuAlaGlnAspSer  4000

AGCCAGATCGAGCTGCTGAAGGAGCTGCTGGATCTGCAGAAGGACATGGTGGTGATGTTGCTGTCGCTACTAGAAGGGAACGTGGTGAACGGCATGATCGCCCGGCAGATGGTGGACATG 12120
SerGlnIleGluLeuLeuLysGluLeuLeuAspLeuGlnLysAspMetValValMetLeuLeuSerLeuLeuGluGlyAsnValValAsnGlyMetIleAlaArgGlnMetValAspMet  4040

CTCGTGGAATCCTCATCCAATGTGGAGATGATCCTCAAGTTCTTCGACATGTTCCTGAAACTCAAGGACATTGTGGGCTCTGAAGCCTTCCAGGACTACGTAACGGATCCCCGTGGCCTC 12240
LeuValGluSerSerSerAsnValGluMetIleLeuLysPhePheAspMetPheLeuLysLeuLysAspIleValGlySerGluAlaPheGlnAspTyrValThrAspProArgGlyLeu  4080

ATCTCCAAGAAGGACTTCCAGAAGGCCATGGACAGCCAGAAGCAGTTCAGCGGTCCAGAAATCCAGTTCCTGCTTTCGTGCTCCGAAGCGGATGAGAACGAAATGATCAACTGCGAAGAG 12360
IleSerLysLysAspPheGlnLysAlaMetAspSerGlnLysGlnPheSerGlyProGluIleGlnPheLeuLeuSerCysSerGluAlaAspGluAsnGluMetIleAsnCysGluGlu  4120

TTCGCCAACCGCTTCCAGGAGCCAGCACGCGACATCGGCTTCAACGTGGCGGTGCTGCTGACCAACCTGTCGGAGCATGTGCCGCATGACCCTCGCCTGCACAACTTCCTGGAGCTGGCC 12480
PheAlaAsnArgPheGlnGluProAlaArgAspIleGlyPheAsnValAlaValLeuLeuThrAsnLeuSerGluHisValProHisAspProArgLeuHisAsnPheLeuGluLeuAla  4160

GAGAGCATCCTTGAGTACTTCCGCCCCTACCTGGGCCGCATCGAGATCATGGGCGCGTCACGCCGCATCGAGCGCATCTACTTCGAGATCTCAGAGACCAACCGCGCCCAGTGGGAGATG 12600
GluSerIleLeuGluTyrPheArgProTyrLeuGlyArgIleGluIleMetGlyAlaSerArgArgIleGluArgIleTyrPheGluIleSerGluThrAsnArgAlaGlnTrpGluMet  4200

CCCCAGGTGAAGGAGTCCAAGCGCCAGTTCATCTTCGACGTGGTGAACGAGGGCGGCGAGGCTGAGAAGATGGAGCTCTTCGTGAGTTTCTGCGAGGACACCATCTTCGAGATGCAGATC 12720
ProGlnValLysGluSerLysArgGlnPheIlePheAspValValAsnGluGlyGlyGluAlaGluLysMetGluLeuPheValSerPheCysGluAspThrIlePheGluMetGlnIle  4240

GCCGCGCAGATCTCGGAGCCCGAGGCCGAGCCGGAGACCGACGAGGACGAGGGCGCGGGCGCCGGCGGAGGCGGCGCCGAAGGCGCGGAGGAGGGCGCGGCGGGGCTCGAGGGCACGGCG 12840
AlaAlaGlnIleSerGluProGluGlyGluProGluThrAspGluAspGluGlyAlaGlyAlaAlaGluAlaGlyAlaGluGlyAlaGluGluGlyAlaAlaGlyLeuGluGlyThrAla  4280

GCCACGGCGGCGGCGGGGGCGACGGCGCGGGTTGTGGCGGCCGCAGGCCCGGGCCCTGCGAGGCCTCAGCTACCGCAGCCTGCGGCGGCGCGTGCGGCGGCTGCGGCGGCTTACGGCCCGC 12960
AlaThrAlaAlaAlaGlyAlaThrAlaArgValValAlaAlaAlaGlyArgAlaLeuArgGlyLeuSerTyrArgSerLeuArgArgArgValArgArgLeuArgArgLeuThrAlaArg  4320

GAGGCGGCCACCGCAGTGGCGGCGCTGCTCTGGGCAGCAGTGACGCGCGCTGGGGCCGCTGGCGCGGGGGCGGCGGCGGGCGCCGCTGGCCTGCTCTGGGGCTCGCTGTTCGGCGGCGGC 13080
GluAlaAlaThrAlaValAlaAlaLeuLeuTrpAlaAlaValThrArgAlaGlyAlaAlaGlyAlaGlyAlaAlaAlaGlyAlaLeuGlyLeuLeuTrpGlySerLeuPheGlyGlyGly  4360

CTGGTGGAGGGCGCCAAGAAGGTGACGGTGACCGAGCTCCTGGCAGGCATGCCCGACCCCACCAGCGACGAGGTGCACGGCGAGCAGCCGGCCGGGCCGGGCGGAGACGCAGACGGCGAG 13200
LeuValGluGlyAlaLysLysValThrValThrGluLeuLeuAlaGlyMetProAspProThrSerAspGluValHisGlyGluGlnProAlaGlyProGlyGlyAspAlaAspGlyGlu  4400

GGTGCCAGCGAGGGCGCTGGAGACGCCGCGGAGGGCGCTGGAGACGAGGAGGAGGCGGTGCACGAGGCCGGGCCGGGCGGTGCCGACGGGGCGGTGGCCGTGACCGATGGGGGCCCCTTC 13320
GlyAlaSerGluGlyAlaGlyAspAlaAlaGluGlyAlaGlyAspGluGluGluAlaValHisGluAlaGlyProGlyGlyAlaAspGlyAlaValAlaValThrAspGlyGlyProPhe  4440

CGGCCCGAAGGGGCTGGCGGTCTCGGGGACATGGGGGACACGACGCCTGCGGAACCGCCCACACCCGAGGGCTCTCCCATCCTCAAGAGGAAATTGGGGGTGGATGGAGTGGAGGAGGAG 13440
ArgProGluGlyAlaGlyGlyLeuGlyAspMetGlyAspThrThrProAlaGluProProThrProGluGlySerProIleLeuLysArgLysLeuGlyValAspGlyValGluGluGlu  4480

CTCCCGCCAGAGCCAGAGCCCGAGCCGGAACCAGAGCTGGAGCCGGAGAAAGCCGATGCCGAGAATGGGGAGAAGGAAGAAGTTCCCGAGCCCACACCAGAGCCCCCCAAGAAGCAAGCA 13560
LeuProProGluProGluProGluProGluProGluLeuGluProGluLysAlaAspAlaGluAsnGlyGluLysGluGluValProGluProThrProGluProProLysLysGlnAla  4520
```

FIG. 2H.

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CCTCCCTCACCCCCTCCAAAGAAGGAGGAAGCTGGAGGCGAATTCTGGGGCAGAACTGGAGGTGCAGAGGGTGAAGTTCCTGAACTACCTGTCCCGGAACTTTTACACCCTGCGGTTCCTT  13680
ProProSerProProProLysLysGluGluAlaGlyGlyGluPheTrpGlyGluLeuGluValGlnArgValLysPheLeuAsnTyrLeuSerArgAsnPheTyrThrLeuArgPheLeu  4560

GCCCTCTTCTTGGCATTTGCCATCAACTTCATCTTGCTGTTTTATAAGGTCTCAGACTCTCCACCAGGGGAGGACGACATGGAAGGCTCAGCTGCTGGGGATGTGTCAGGTGCAGGCTCT  13800
AlaLeuPheLeuAlaPheAlaIleAsnPheIleLeuLeuPheTyrLysValSerAspSerProProGlyGluAspAspMetGluGlySerAlaAlaGlyAspValSerGlyAlaGlySer  4600

GGTGGCAGCTCTGGCTGGGGCTTGGGGGCCCGGAGAGGAGGCAGAGGGCGATGAGGATGAGAACATGGTGTACTACTTCCTGGAGGAAAGCACAGGCTACATGGAACCCGCCCTGCGGTGT  13920
GlyGlySerSerGlyTrpGlyLeuGlyAlaGlyGluGluAlaGluGlyAspGluAspGluAsnMetValTyrTyrPheLeuGluGluSerThrGlyTyrMetGluProAlaLeuArgCys  4640

CTGAGCCTCCTGCATACACTGGTGGCCTTTCTCTGCATCATTGGCTATAATTGTCTCAAGGTGCCCCTGGTAATCTTTAAGCGGGAGAAGGAGCTGGCCCCGGAAGCTGGAGTTTGATGGC  14040
LeuSerLeuLeuHisThrLeuValAlaPheLeuCysIleIleGlyTyrAsnCysLeuLysValProLeuValIlePheLysArgGluLysGluLeuAlaArgLysLeuGluPheAspGly  4680

CTGTACATCACGGAGCAGCCTGAGGACGATGACGTGAAGGGGCAGTGGGACCGACTGGTGCTCAACACGCCGTCTTTCCCTAGCAACTACTGGGACAAGTTTGTCAAGCGCAAGGTCCTG  14160
LeuTyrIleThrGluGlnProGluAspAspAspValLysGlyGlnTrpAspArgLeuValLeuAsnThrProSerPheProSerAsnTyrTrpAspLysPheValLysArgLysValLeu  4720

GACAAACATGGGGACATCTACGGGCGGGAGCGGATTGCTGAGCTACTGGGCATGGACCTGGCCACACTAGAGATCACAGCCCACAATGAGCGCAAGCCCAACCCGCCGCCAGGGCTGCTG  14280
AspLysHisGlyAspIleTyrGlyArgGluArgIleAlaGluLeuLeuGlyMetAspLeuAlaThrLeuGluIleThrAlaHisAsnGluArgLysProAsnProProProGlyLeuLeu  4760

ACCTGGCTCATGTCCATCGATGTCAAGTACCAGATCTGGAAGTTCGGGGTCATCTTCACAGACAACTCCTTCCTGTACCTGGGCTGGTATATGGTGATGTCCCTCTTGGGACACTACAAC  14400
ThrTrpLeuMetSerIleAspValLysTyrGlnIleTrpLysPheGlyValIlePheThrAspAsnSerPheLeuTyrLeuGlyTrpTyrMetValMetSerLeuLeuGlyHisTyrAsn  4800

AACTTCTTCTTTGCTGCCCATCTCCTGGACATCGCCATGGGGGTCAAGACGCTGCGCACCATCCTGTCCTCTGTCACCCACAATGGGAAACAGCTGGTGATGACCGTGGGCCTTCTGGCG  14520
AsnPhePhePheAlaAlaHisLeuLeuAspIleAlaMetGlyValLysThrLeuArgThrIleLeuSerSerValThrHisAsnGlyLysGlnLeuValMetThrValGlyLeuLeuAla  4840

GTGGTCGTCTACCTGTACACCGTGGTGGCCTTCAACTTCTTCCGCAAGTTCTACAACAAGAGCGAGGATGAGGATGAACCTGACATGAAGTGTGATGACATGATGACGTGTTACCTGTTT  14640
ValValValTyrLeuTyrThrValValAlaPheAsnPhePheArgLysPheTyrAsnLysSerGluAspGluAspGluProAspMetLysCysAspAspMetMetThrCysTyrLeuPhe  4880

CACATGTACGTGGGTGTCCGGGCTGGCGGAGGCCATTGGGGACGAGATCGAGGACCCCGCCGGGTGACGAATACGAGCTCTACAGGGTGGTCTTCGACATCACCTTCTTCTTCTTCGTCATC  14760
HisMetTyrValGlyValArgAlaGlyGlyGlyIleGlyAspGluIleGluAspProAlaGlyAspGluTyrGluLeuTyrArgValValPheAspIleThrPhePhePhePheValIle  4920

GTCATCCTGTTGGCCATCATCCAGGGTCTGATCATCGACGCTTTTGGTGAGCTCCGAGACCAACAAGAGCAAGTGAAGGAGGATATGGAGACCAAGTGCTTCATCTGTGGAATCGGCAGT  14880
ValIleLeuLeuAlaIleIleGlnGlyLeuIleIleAspAlaPheGlyGluLeuArgAspGlnGlnGluGlnValLysGluAspMetGluThrLysCysPheIleCysGlyIleGlySer  4960

GACTACTTTGATACGACACCGCATGGCTTCGAGACTCACACGCTGGAGGAGCACAACCTGGCCAATTACATGTTTTTCCTGATGTATTTGATAAACAAGGATGAGACAGAACACACGGGT  15000
AspTyrPheAspThrThrProHisGlyPheGluThrHisThrLeuGluGluHisAsnLeuAlaAsnTyrMetPhePheLeuMetTyrLeuIleAsnLysAspGluThrGluHisThrGly  5000

CAGGAGTCTTATGTCTGGAAGATGTACCAAGAGAGATGTTGGGATTTCTTCCCAGCTGGTGATTGTTTCCGTAAGCAGTATGAGGACCAGCTTAGCTGACACACCCCCAGCTGGCCCTCC  15120
GlnGluSerTyrValTrpLysMetTyrGlnGluArgCysTrpAspPhePheProAlaGlyAspCysPheArgLysGlnTyrGluAspGlnLeuSer***                          5032

ACCCCCACCTCAAGTGCCTTATTCTCACAGCAAGCCCCTTAGTCCCCAAGCCCCTCCCCCTAAGGCAGCTGGGGGAGAGGTGACCTAGTACTGGAAAATAAATCTGTCGTACGCCCCCCA  15240
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FIG.3.

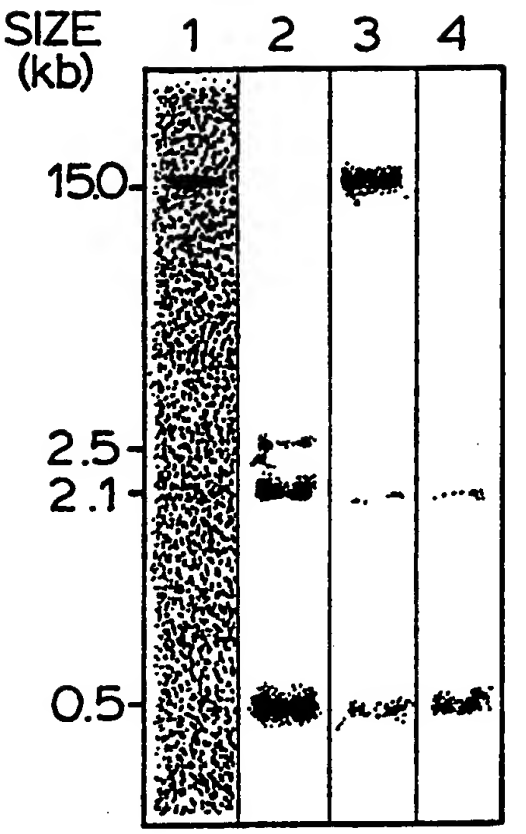


FIG.4.

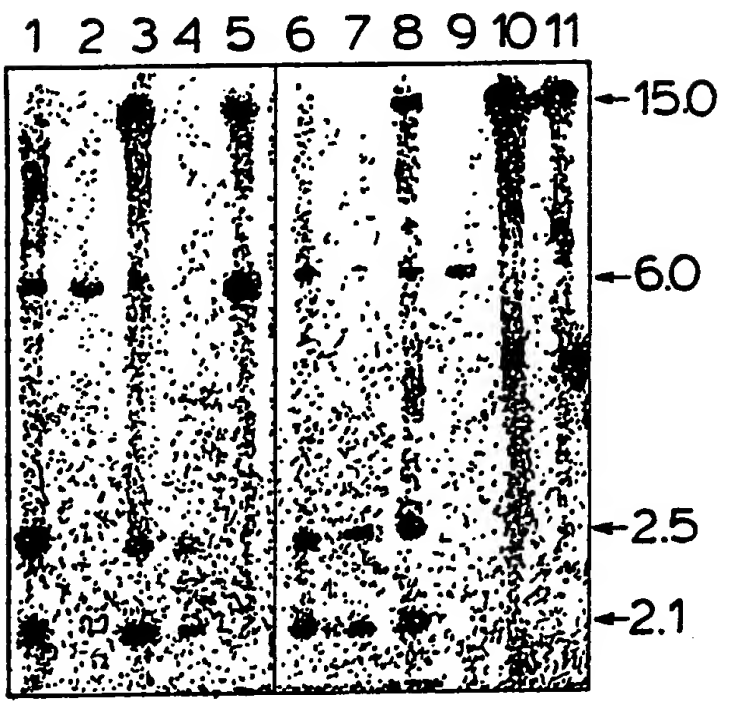
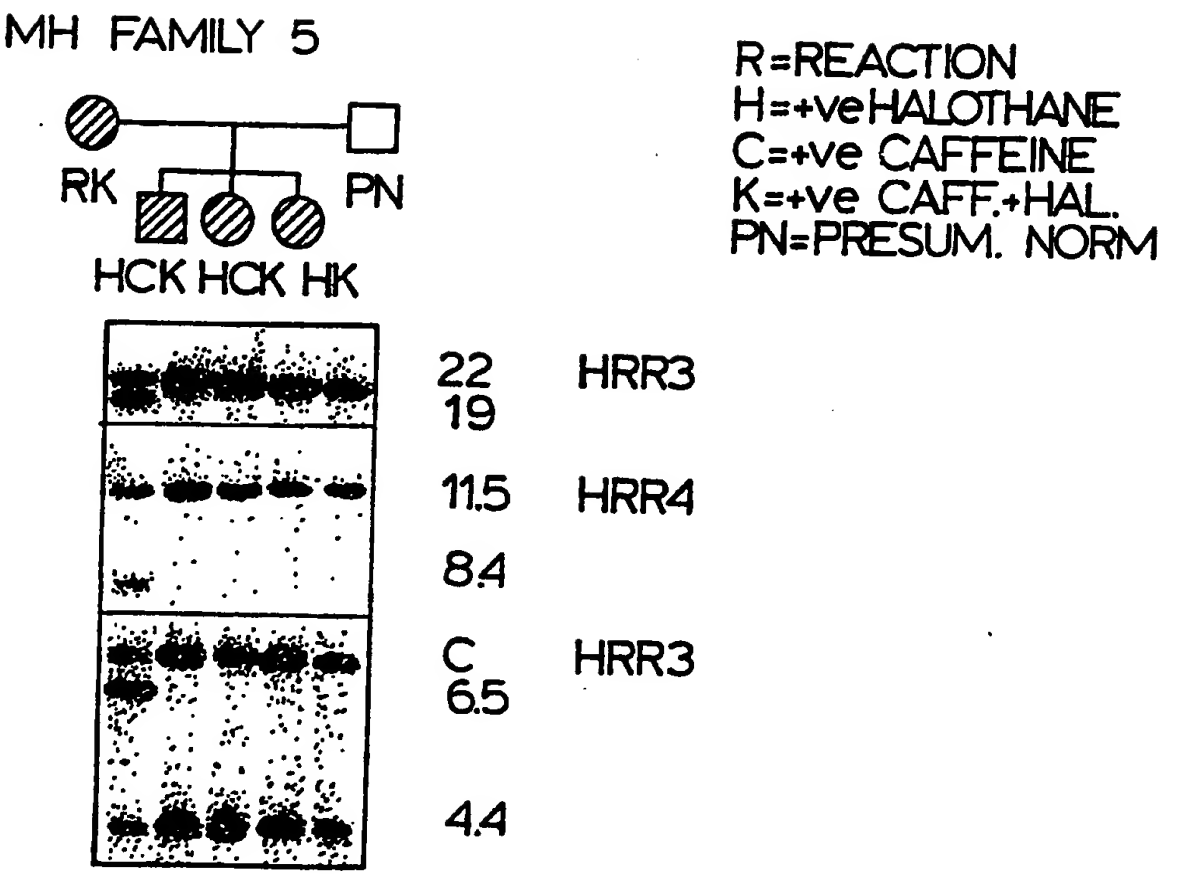


FIG.5A.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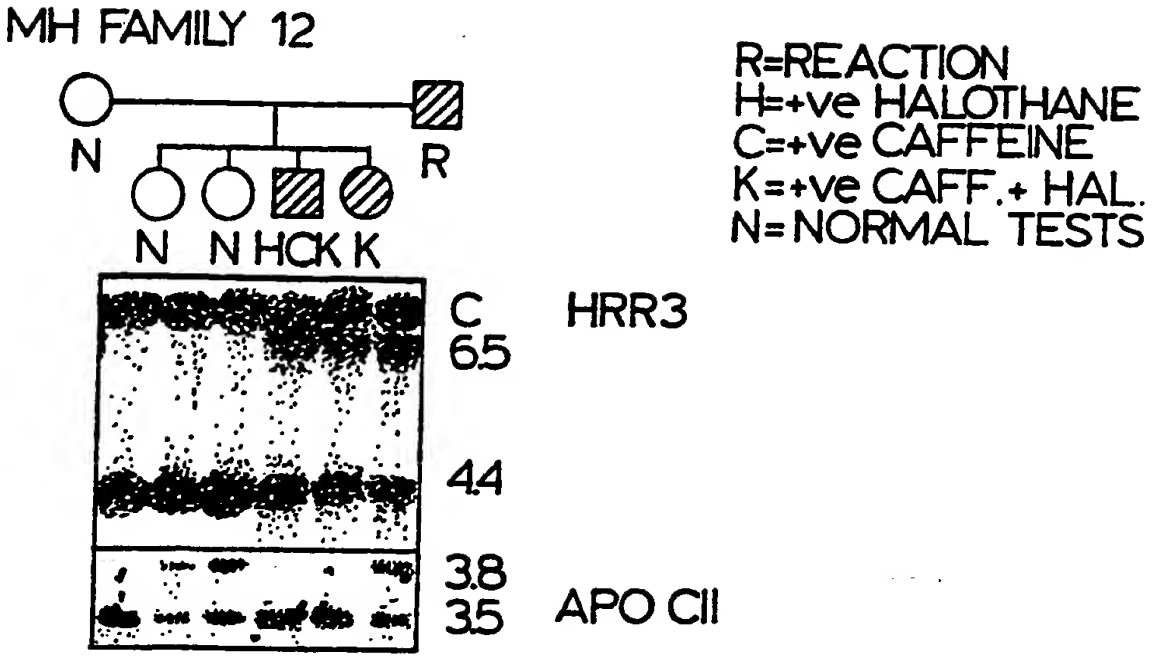
RK
PN
HCK HCK HK
R=REACTION
H=+ve HALOTHANE
C=+ve CAFFEINE
K=+ve CAFF.+HAL.
PN=PRESUM. NORM
22
19
HRR3
11.5
HRR4
8.4
C
6.5
HRR3
4.4

FIG.5B.


MH FAMILY 12
N
R
N N HCK K
R=REACTION
H=+ve HALOTHANE
C=+ve CAFFEINE
K=+ve CAFF.+ HAL.
N=NORMAL TESTS
C
6.5
HRR3
4.4
3.8
3.5
APO CII

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00312

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/12, C 12 P 21/02, C 12 P 21/08, C 07 K 13/00, C 12 Q 1/68, G 01 N 33/50

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, C 12 N, C 12 P, G 01 N, C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	Nature, vol. 339, 8 June 1989, (London, GB), H. Takeshima et al.: "Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor", pages 439-445 see the whole article cited in the application --	1
X	Journal of Biological Chemistry, vol. 264, no. 3, 25 January 1989, (Baltimore, US), G. Meissner et al.: "Structural and functional correlation of the trypsin-digested Ca2+ release channel of skeletal muscle sarcoplasmic reticulum", pages 1715-1722 see the whole article cited in the application --	13,14

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29th November 1990	24 JAN 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Chemical Abstracts, vol. 107, no. 1, 6 July 1987, (Columbus, Ohio, US), K.P. Campbell et al.: "Identification and characterization of the high affinity (3H)ryanodine receptor of the junctional sarcoplasmic reticulum calcium release channel", see page 271, abstract 2874q, & J. Biol. Chem. 1987, 262(14), 6460-3	13,15
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P,X	Journal of Biological Chemistry, vol. 265, no. 4, 5 February 1990, (Baltimore, US), F. Zorzato et al.: "Molecular cloning of cDNA encoding human and rabbit forms of the Ca2+ release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum", pages 2244-2256 see the whole article	1-12,17
